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Tissue specificity of mitochondrial F_0F_1-ATPase activity of *Lilium longiflorum* plant

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Abstract A large difference was found in the activities of oligomycin-sensitive mitochondrial F_0F_1-ATPase isolated from different tissues of *Lilium longiflorum* plants. The enzyme activity of F_0F_1-ATPase in pollen was the highest, while that in bulbs was the lowest. When ATPases were cross-reconstituted from F_1-ATPases and F_1-depleted submitochondrial particles (SMP), ATPases reconstituted from F_1-depleted pollen SMP showed the higher activity regardless of the source of F_1-ATPase. Fatty acid compositions of phospholipids in SMP were also different between bulbs and pollen. These suggest that the F_0 portion and/or its environment are important for regulation of F_0F_1-ATPase activity in *L. longiflorum* plant.

Key words: F_0F_1-ATPase; Tissue specificity; Submitochondrial particle; Reconstitution; Lipid composition; *Lilium longiflorum*

1. Introduction

Mitochondrial F_0F_1-ATPase plays a crucial role in the oxidative phosphorylation in eukaryote cells. The enzyme consists of two sectors; a hydrophilic F_1 portion containing the catalytic site, and a hydrophobic membranous F_0 portion constituting a proton channel. F_1-ATPase has been purified from a variety of plant sources [1–5], which has revealed that the subunit composition of F_1-ATPase is rather common. However, little information is available concerning regulatory factors of plant F_0F_1-ATPase compared with those of mammals and yeast [6,7], since F_0F_1-ATPase has been purified only from spinach leaves [8]. It was recently reported that ATP hydrolysis activity of isolated plant mitochondria was controlled in a tissue-specific manner [9] but there was no indication of which component controlled the activity. The present communication describes that F_0F_1-ATPase of SMP isolated from different tissues of *Lilium longiflorum* plants shows different enzyme activities and that the tissue specificity concerns the F_0 portion embedded in the membrane.

2. Materials and methods

2.1. Plant materials

Bulbs of *Lilium longiflorum* Thunb. cv. Hinomoto were purchased locally and stored in a refrigerator until use. Mature pollen, anther walls and leaves were collected from *L. longiflorum* plants obtained at a local market. Pollens were rinsed with petroleum ether to remove pigments from the surface, and stored in a refrigerator until use.

2.2. Preparation of SMP

Mitochondria were isolated isotonically and then further purified on a Percoll gradient by the method described previously [10] except for the use of EDTA instead of EGTA. The obtained mitochondrial pellet was suspended in 0.4 M mannitol, 1 mM EDTA, 0.5 mM PMSF, and 10 mM Tricine-KOH, pH 7.2, and disrupted by a sonification (2×1 min) at a maximum output (23 kHz, 50 W). Unbroken mitochondria were removed by centrifugation at $10,000 \times g$ for 10 min and SMP were then collected by centrifugation at $100,000 \times g$ for 60 min. All above procedures were carried out at 4°C.

2.3. Purification of F_1-ATPase

F_1-ATPase was dissolved out from SMP suspended in 1 mM EDTA, 4 mM ATP, 1 mM PMSF and 20 mM TES-KOH, pH 7.5, using CH_2Cl_2 instead of $CHCl_3$ and further purified to homogeneity at room temperature by the method described previously [5]. The enzyme was dissolved in 0.25 M sucrose, 1 mM ATP, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, and stored at −80°C until use.

2.4. Preparation of F_1-depleted SMP

F_1-depleted SMP were prepared essentially by the method described previously [11]. SMP were suspended in 0.25 M sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 8.0, at 10 mg protein per ml. 1 vol. of SMP suspension was mixed with 1 vol. of 100 mM Tris-HCl, pH 8.0, containing 4 M urea, 4 mM EDTA, and incubated at 0°C for 30 min. After the incubation, the suspension was centrifuged at $100,000 \times g$ for 30 min. The pellet was washed once with 0.25 M sucrose, 10 mM Tris-HCl, pH 7.5. The resultant pellet was suspended in 0.25 M sucrose, 5 mM dithiothreitol, pH 7.5, at 10 mg protein per ml and served as F_1-depleted SMP.

2.5. Reconstitution of oligomycin-sensitive F_0F_1-ATPase

F_1-depleted SMP (100 μg protein) were added to the purified F_1-ATPase (10 μg protein) and incubated for 30 min at 25°C, adjusting the volume to 100 μl with the medium containing in final concentrations 0.25 M sucrose, 20 mM $(CH_3COO)_2Mg$ and 10 mM Tris-HCl, pH 8.0 [11]. The incubation mixture was centrifuged at $100,000 \times g$ for 15 min to remove unbound F_1-ATPase. The pellet was washed once with 0.25 M sucrose and 10 mM Tris-HCl, pH 8.0. The resultant pellet was suspended in 0.25 M sucrose and 10 mM Tris-HCl, pH 8.0, and served as the reconstituted ATPase.

2.6. ATPase assay

ATPase activity was assayed colorimetrically by measuring P_i liberated [5]. A reaction mixture (1 ml) contained 3 mM ATP, 3 mM $MgCl_2$, 20 mM MOPS-Tris, pH 8.0, and enzyme solution. Sucrose (0.25 M) was added to the mixture when SMP were used as the enzyme source. The reaction mixture without $MgCl_2$ was employed as a blank. The enzyme activity was represented as Mg-dependent ATPase activity. The enzyme activity in the presence of oligomycin (10 μg/ml) was also measured to confirm oligomycin sensitivity.

2.7. ELISA

The F_1-ATPase purified from *L. longiflorum* bulbs was used as the antigen for immunization of rabbits. After repeated booster injections, rabbits were bled and an IgG fraction was prepared from antisera by $(NH_4)_2SO_4$ fractionation (25–33%). The antibody obtained showed

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Abbreviations: CL, cardiolipin; ELISA, enzyme linked immunosorbant assay; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMSF, phenylmethyl sulfonylfluoride; SMP, submitochondrial particles; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid.

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equal factors both to bulb and pollen F_1-ATPase. ELISA was applied to quantitate F_0F_1-ATPase on the surface of SMP using the above antibody. A horseradish peroxidase-conjugated anti-rabbit IgG was employed for measurement of the cross-reaction [12].

2.8. Protein determination

Protein was measured using Comassie brilliant blue G-250 with bovine serum albumin as a standard [13].

2.9. Lipid analysis

Lipids were extracted from SMP isolated from bulbs and pollen [14]. PC, PE and CL were separated with Si TLC [15] and transmethylated with H_2SO_4-MeOH [14]. Methyl esters of fatty acids were analyzed by GLC with Silar 10 C column (3 mm × 3 m).

3. Results and discussion

3.1. F_0F_1-ATPase activity of SMP

When SMP were prepared from different tissues of *L. longiflorum* plants and served as an enzyme source, ATPase acitivity was detected (Table 1). Oligomycin and NaN_3 inhibited about 80% and 50% of the enzyme activities of the SMP, respectively, while additiion of vanadate and KNO_3 did not significantly reduce the enzyme activities. The majority of the ATPases present in the SMP fraction is, thus, concluded to be mitochondrial F_0F_1-ATPase. Pollen SMP showed the highest ATPase activity among SMP prepared from various tissues, while the activity of bulb SMP was the lowest; the ratio of the enzyme activity of pollen SMP to that of bulb SMP was more than 10 (Table 1). The enzyme activities of anther wall and leaf SMP ranged between those of bulb and pollen SMP. These findings indicate that mitochondrial F_0F_1-ATPase shows a tissue-specific activity. There are two possibilities to explain this; (i) a difference in the number of the ATPase molecules in mitochondria, and (ii) the occurrence of regulatory factors in mitochondria.

We estimated the number of the ATPase molecule on the surface of SMP by ELISA, using the antibody raised against bulb F_1-ATPase. The result showed that numbers of F_1-ATPase in SMP isolated from bulbs and pollen were the same (Fig. 1). Therefore, the enzyme activity would be regulated in a tissue-specific manner by unknown regulatory factors. Recently Valerio et al. [9] reported tissue specificity of ATP hydrolysis by isolated plant mitochondria, leaf mitochondria from pea, and tuber ones from potato. Our present results clearly indicate that F_0F_1-ATPase is regulated in a tissue-specific manner even in the same plant.

A question has been raised as to which part, the F_1- or F_0 portion, of F_0F_1-ATPase concerns the enzyme activity. To evaluate the contribution of F_1-ATPase to the tissue specificity, F_1-ATPase was purified to homogeneity from bulb and pollen SMP (Table 2) [5]. However, we could not explain the more than 10 times difference in F_0F_1-ATPase activities of bulbs and pollen, although the activity of pollen F_1-ATPase was twice as high as that of the bulb.

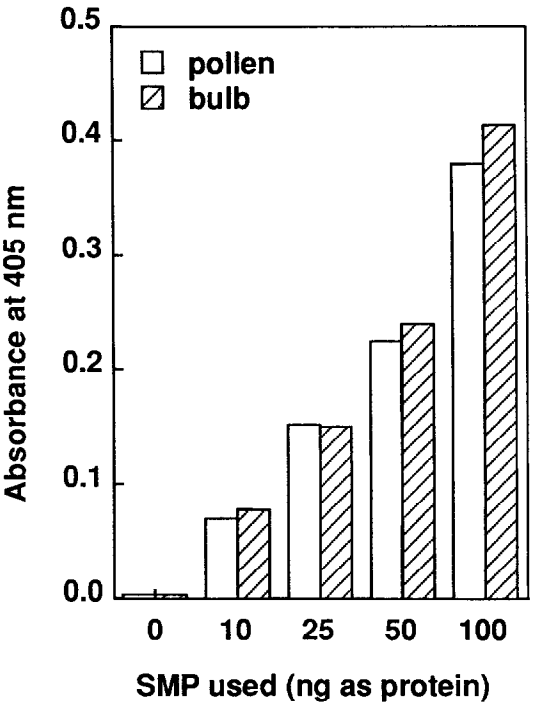

Fig. 1. Quantification of F_0F_1-ATPase on the surface of SMP by ELISA. The same amounts of bulb and pollen SMP protein were used for cross-reaction to the antibody raised against the bulb F_1-ATPase.

3.2. Reconstitution of ATPase from F_1-depleted SMP and F_1-ATPase

Recently oligomycin-sensitive F_0F_1-ATPase was reconstituted from F_1-depleted SMP and isolated F_1-ATPase [11]. To evaluate the contribution of the F_0 portion to the tissue specificity of F_0F_1-ATPase activity, oligomycin-sensitive ATPases were cross-reconstituted from F_1-depleted SMP and purified F_1-ATPases of *L. longiflorum* plants. When we treated SMP with 2 M urea in the presence of 2.5 mM EDTA, F_1-depleted SMP was obtained (Table 2). After the incubation of F_1-depleted SMP with F_1-ATPase, reconstituted ATPase was obtained. The

Table 1
ATPase activity of SMP isolated from different tissues of *L. longiflorum* plant and effects of inhibitors on the enzyme activity

Origin of SMP	ATPase activity (nkat/mg protein)				
	None	Oligomycin (10 μg/ml)	NaN_3 (50 mM)	Vanadate (100 μM)	KNO_3 (30 mM)
Bulb	0.56	0.11	0.28	0.42	0.45
	(100)	(20)	(51)	(75)	(81)
Leaf	4.98	1.68	2.84	4.21	3.98
	(100)	(34)	(57)	(85)	(80)
Anther wall	1.67	0.36	0.73	1.39	1.35
	(100)	(22)	(44)	(83)	(81)
Pollen	9.67	2.71	5.31	8.21	8.37
	(100)	(28)	(55)	(85)	(87)

Figures in parentheses represent values relative to those without inhibitors.

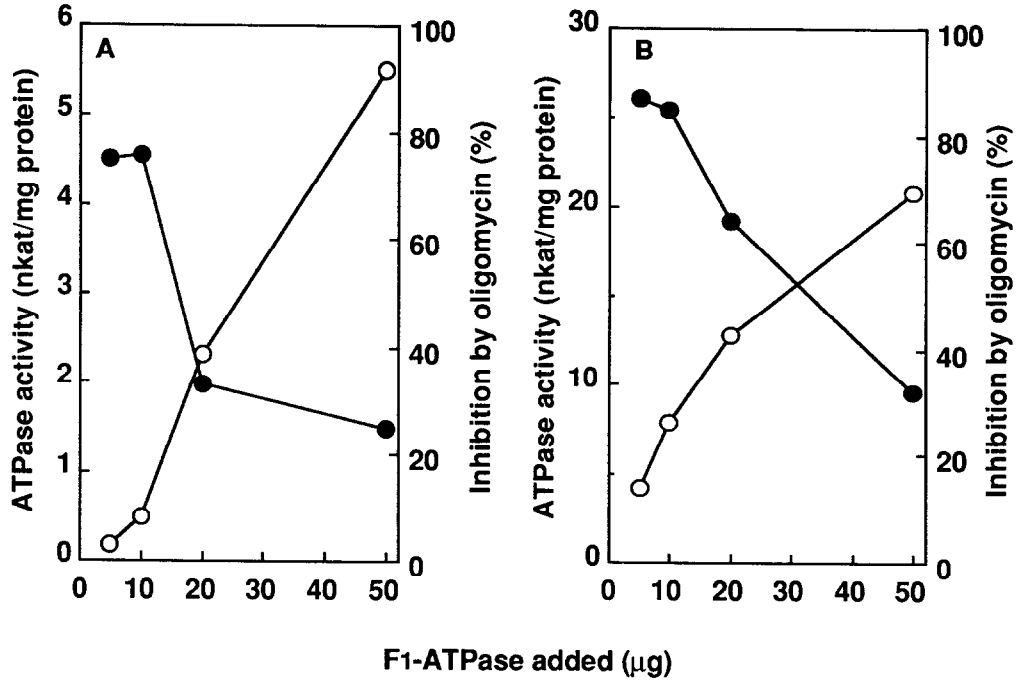

Fig. 2. Effects of amounts of F_1-ATPase added on the enzyme activity and oligomycin sensitivity of reconstituted ATPase. Different amounts of purified F_1-ATPase were incubated with F_1-depleted SMP (100 μg protein) as described in section 2.5. ATPase activity was measured in the absence and presence of oligomycin (10 μg/assay). (A) ATPase reconstituted from F_1-ATPase and F_1-depleted SMP of bulbs; (B) ATPase reconstituted from F_1-ATPase and F_1-depleted SMP of pollen. ATPase activity (○) was represented as nkat per mg protein of reconstituted ATPase. Inhibition of ATPase activity by oligomycin (●) was represented as % inhibition.

activity of homologously reconstituted ATPase was increased with an increase in the amount of F_1-ATPase added in both cases of bulbs and pollen, while oligomycin sensitivity was decreased (Fig. 2). Therefore, the following condition was employed for reconstitution: addition of 10 μg purified F_1-ATPase to F_1-depleted SMP (100 μg protein). The activity of ATPase reconstituted under this condition was inhibited by more than 80% by oligomycin (Fig. 2).

Table 2
Enzyme activity of ATPase reconstituted from F_1-ATPase and F_1-depleted SMP prepared from bulbs and pollen of *L. longiflorum* plant

Component (μg protein)	ATPase activity (nkat/assay)	Inhibition by oligomycin (%)
Bulb F_1-ATPase (10)	0.60	13
Pollen F_1-ATPase (10)	1.3	10
Bulb SMP (100)	0.05	74
Pollen SMP (100)	0.97	86
Bulb F_1-depleted SMP (100)	0.001	0
Pollen F_1-depleted SMP (100)	0.002	0
ATPase reconstituted from bulb F_1-depleted SMP (100) and bulb F_1-ATPase (10)	0.08	84
ATPase reconstituted from bulb F_1-depleted SMP (100) and pollen F_1-ATPase (10)	0.12	79
ATPase reconstituted from pollen F_1-depleted SMP (100) and bulb F_1-ATPase (10)	1.15	81
ATPase reconstituted from pollen F_1-depleted SMP (100) and pollen F_1-ATPase (10)	0.77	92

When bulb and pollen F_1-depleted SMP (100 μg protein) were cross-reconstituted with bulb or pollen F_1-ATPase (10 μg), reconstituted ATPases sensitive to oligomycin were obtained (Table 2). The activities of homologously reconstituted ATPases were the same levels as those of the initial SMP. However, when bulb F_1-depleted SMP were cross-reconstituted with pollen F_1-ATPase, the activity was low. In contrast, when pollen F_1-depleted SMP was cross-reconstituted with bulb F_1-ATPase, the activity was as high as that of the pollen SMP. Since numbers of F_0F_1-ATPases in pollen and bulb SMP were similar, as shown in Fig. 1, these results indicate that the F_0 portion embedded in the membrane contributes to the tissue specificity of the ATPase activity. Several F_0F_1-ATPase inhibitors have been isolated from mammals [16–18] and yeast [19]. It was reported that PVP-like peptide was involved in the F_0 portion of potato tuber F_0F_1-ATPase [11]. An ATPase inhibitor protein (8.3 kDa) was also isolated from potato tuber mitochondria [20]. These earlier findings suggest a possible control of the ATPase activity by inhibitor(s) in higher plants.

3.3. *Fatty acid compositions of phospholipids in SMP*

Major phospholipids (PC, PE and CL) of SMP were isolated and their fatty acid compositions were examined. PC and PE of bulb SMP were composed of 16:0 and 18:2, while 16:0 and 18:3 increased with the decrease in 18:2 in PC and PE of pollen SMP (Table 3). In CL, 18:2 accounted for more than 70% in bulb SMP, but 18:2 and 18:3 were major fatty acid constituents in pollen SMP. Thus, fatty acid composition of major phospholipids in SMP were different between bulb and pollen SMP. These results suggest that the membrane environment in the vicinity of the F_0 portion differs. It is known that the lipid composition of the membrane affects the catalytic properties

Table 3
Fatty acid compositions of PC, PE and CL of SMP isolated from bulbs and pollens of *L. longiflorum* plant

Lipid class	Tissue	Fatty acid composition (%)				
		16:0	18:0	18:1	18:2	18:3
PC	Bulbs	17.0	0.5	2.5	71.9	8.3
	Pollen	35.4	0.7	3.8	25.0	35.2
Pe	Bulbs	27.2	0.4	2.4	66.5	3.5
	Pollen	38.0	0.5	0.9	31.4	29.2
CL	Bulbs	7.6	1.8	2.8	74.1	13.7
	Pollen	11.3	3.9	9.3	31.0	44.5

of mammalian mitochondrial ATPase [21,22]. We, therefore, assume that the ATPase activity of plant mitochondria could also be affected by composition of phospholipids around the enzyme.

As a conclusion, our findings indicate that the membranous F_0 portion and/or its environment of the membrane regulate the tissue specificity of mitochondrial F_0F_1-ATPase activity.

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