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Short Communication

Inhibition of the Activity of NAD(P)H-Dependent Oxidase in Pistils of *Lilium longiflorum* by cAMP

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The effects of exogenous cAMP on the activities of the stress enzymes were studied using the extracts from stigmas and styles of *Lilium longiflorum* cv. Hinomoto without pollination in relation to self-incompatibility. The activity of NADH- and NADPH-dependent oxidases (O_2^--forming enzymes) was inhibited by cAMP. This inhibition was noncompetitive with NAD(P)H.

Key words: cAMP — *Lilium longiflorum* — NAD(P)H-dependent oxidase — Pistil — Pollination — Superoxide (O_2^-).

The pollen tubes of *Lilium longiflorum*, which has a gametophytic self-incompatibiliy system, stop elongating in the stylar canal approximately 20 h after self-incompatible pollination on the surface of stigmas (Hiratsuka et al. 1983, Tezuka et al. 1993). This phenomenon may be associated with the stress caused by the self-incompatible pollination. According to Tezuka et al. (1997), the self-incompatible pollination on stigmas of *L. longiflorum* induces the activation of stress enzymes, such as NADH-dependent and NADPH-dependent oxidases, xanthine oxidase, superoxide dismutase (SOD), catalase and ascorbate peroxidase in the pistils. Takenawa et al. (1986) reported that the increase in the levels of cAMP in Guinea pig neutrophils results in the suppression of O_2^--forming enzyme activity. Gulyaeva et al. (1987) also reported that the amount of O_2^- reacting with SOD is decreased by cAMP. We suppose that cAMP may decrease the levels of O_2^- reacting with SOD in pistils of *L. longiflorum*. Tezuka et al. (1993) also reported that the elongation of pollen tubes after self-incompatible pollination in lily was promoted by exogenous cAMP and the length of tubes after cAMP treatment was similar to that after cross-compatible pollination. In the present study, therefore, we examined the relationship between cAMP and the activity of stress enzymes in pistils of *L. longiflorum* without pollination to analyze the self-incompatibility mechanism.

Lilies (*Lilium longiflorum* Thunb. cv. Hinomoto) were grown in a greenhouse at Nagoya University. Flowers from Hinomoto lilies were used on the day after anthesis. The pistils from the flowers of non-pollinated Hinomoto lilies were separated into stigmas and styles. For preparation of enzyme fraction from stigmas and styles, all subsequent steps were carried out at 0–4°C. The stigmas and styles of Hinomoto lilies were homogenized separately in a glass homogenizer in 3 ml g^{-1} of a grinding medium composed of 0.1 M 3-morpholinopropanesulfonic acid (MOPS)-KOH (pH 7.5), 1 mM disodium-EDTA and 0.5 mM dithiothreitol (DTT). The homogenate was squeezed through a layer of Miracloth (Calbiochem-Novabiochem. Co., San Diego, CA. U.S.A.) and centrifuged for 20 min at 10,000 × *g*. The supernatant was further centrifuged for 60 min at 100,000 × *g*. The resulting pellet, used as a membrane fraction, was dispersed in a small volume of the above-mentioned grinding medium and then sonicated three times at 0°C, for 10 s each time, with a sonicator (model 5202; Ohtake Works Co. Ltd., Tokyo) at an output of 100 W. The supernatant (used as a soluble fraction) after centrifugation at 100,000 × *g* and the membrane fraction after sonication were separately dialyzed against 25 mM MOPS-KOH (pH 7.5) that contained 0.1 mM disodium-EDTA and 0.1 mM DTT for 4 h at 4°C and used for assay of the activities of NADH-dependent oxidase, NADPH-dependent oxidase, xanthine oxidase, SOD, catalase and ascorbate peroxidase (Tezuka et al. 1997). The activities of enzymes were estimated at 25°C with a spectrophotometer (model U-3210; Hitachi, Tokyo). SOD was assayed by the method of Asada et al. (1973), NADH- and NADPH-dependent oxidases by the method of Azzi et al. (1975), xanthine oxidase by the method of Hashimoto (1974), and catalase by the method of Beers and Sizer (1952). Ascorbate peroxidase was assayed at 25°C in a reaction mixture (1 ml) that contained 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbate, 0.1 mM H_2O_2 and an enzyme fraction by a modified version of the method of Tanaka et al. (1982). Protein was quantitated by the method of Lowry et al. (1951), with bovine serum albumin as the standard. All experiments were repeated three times with similar results and only representative results are

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shown here.

The activities of active oxygen species-forming and -scavenging enzymes in membrane and soluble fractions prepared separately from stigmas and styles of non-pollinated Hinomoto lilies were not significantly affected by the addition of cAMP into the reaction mixture at various concentrations (0, 0.01, 0.1, 1 and 10 μM), except for NADH- and NADPH-dependent oxidases which are active oxygen species-forming enzymes and as representative results, the effects of cAMP at 0.1 μM on the enzymes are shown in Table 1. In the case of NAD(P)H-dependent oxidases, both the membrane and the soluble fractions from stigmas and styles contained not only NADH-dependent oxidase but also NADPH-dependent oxidase, and the activities of both NADH- and NADPH-dependent oxidases in membrane fractions were significantly decreased by cAMP at 0.1 μM, but those in soluble fractions were not (Table 1).

The dose-responses to NADH and NADPH of the activities of NADH- and NADPH-dependent oxidases, respectively, in the membrane fraction obtained from stigmas and from styles of non-pollinated Hinomoto lilies in the presence and absence of cAMP at 0.1 μM exhibited a hyperbolic curve either in the presence or absence of cAMP (data not shown). Double-reciprocal plots of the activities of both NADH- and NADPH-dependent oxidases versus NADH and NADPH concentrations (Fig. 1), respectively, were obtained in the presence and absence of cAMP obtained from the dose-response curves. The K_m, V_{max} and K_i values of NADH-dependent oxidase for NADH in the membrane fractions from stigmas were 10.5 μM, 1.0 μmol (mg protein)$^{-1}$ min^{-1} and 0.3 μM, respectively, and those of NADPH-dependent oxidase for NADPH were 18.4 μM, 1.5 μmol (mg protein)$^{-1}$ min^{-1} and 0.2 μM, respectively. Furthermore, these values in the membrane fractions from styles were 13.8 μM, 1.1 μmol (mg protein)$^{-1}$ min^{-1} and 0.4 μM for NADH, and 25.0 μM, 1.6 μmol (mg protein)$^{-1}$ min^{-1} and 0.1 μM for NADPH, respectively. In kinetic analysis shown in Figure 1, cAMP gave a noncompetitive inhibitory effect on NADH- and NADPH-dependent oxidases.

On the other hand, the dose-response to NADH and NADPH for NADH- and NADPH-dependent oxidases in the soluble fractions from stigmas and styles of Hinomoto lilies was different from those in the membrane fractions. The dose-response of both enzymes in the soluble fractions

Table 1 The effects of cAMP on the activities of NADH- and NADPH-dependent oxidase, xanthine oxidase, superoxide dismutase, catalase and ascorbate peroxide in the membrane and soluble fractions from stigmas and styles of *Lilium longiflorum* in the absence and presence of cAMP

	Stigmas		Styles	
	Membrane	Soluble	Membrane	Soluble
NADH-oxidase [μmol (mg protein)$^{-1}$]				
without cAMP	0.77±0.06	2.17±0.04	0.95±0.09	0.97±0.03
with cAMP	0.54±0.00**	2.18±0.05	0.74±0.09**	0.96±0.01
NADPH-oxidase [μmol (mg protein)$^{-1}$]				
without cAMP	1.19±0.06	2.30±0.11	1.05±0.04	0.56±0.02
with cAMP	0.79±0.11**	2.37±0.15	0.56±0.02**	0.56±0.12
Xanthine oxidase [μmol (mg protein)$^{-1}$]				
without cAMP	6.06±0.33	1.66±0.04	6.08±1.00	2.29±0.23
with cAMP	5.73±0.10	1.85±0.32	7.02±0.65	2.44±0.07
Superoxide dismutase [units (mg protein)$^{-1}$]				
without cAMP	2.44±0.22	1.50±0.08	4.61±0.37	3.68±0.12
with cAMP	2.36±0.11	1.48±0.08	4.37±0.08	3.68±0.12
Catalase [μmol (mg protein)$^{-1}$]				
without cAMP	176.63±0.22	170.73±3.48	169.04±4.27	213.01±2.25
with cAMP	177.99±4.08	172.47±1.74	166.19±4.26	213.01±6.73
Ascorbate peroxidase [μmol (mg protein)$^{-1}$]				
without cAMP	42.70±3.88	385.77±9.45	36.53±4.06	624.60±5.13
with cAMP	42.70±0.09	373.32±8.05	38.56±2.03	623.61±9.08

Each value is the mean±SD of results from 5 replicates. The asterisks indicate significant differences in P values between the activities of NADH- or NADPH-dependent oxidase in the absence and presence of cAMP at less than 0.01 (**) by t-tests. cAMP concentration: 0.1 μM.

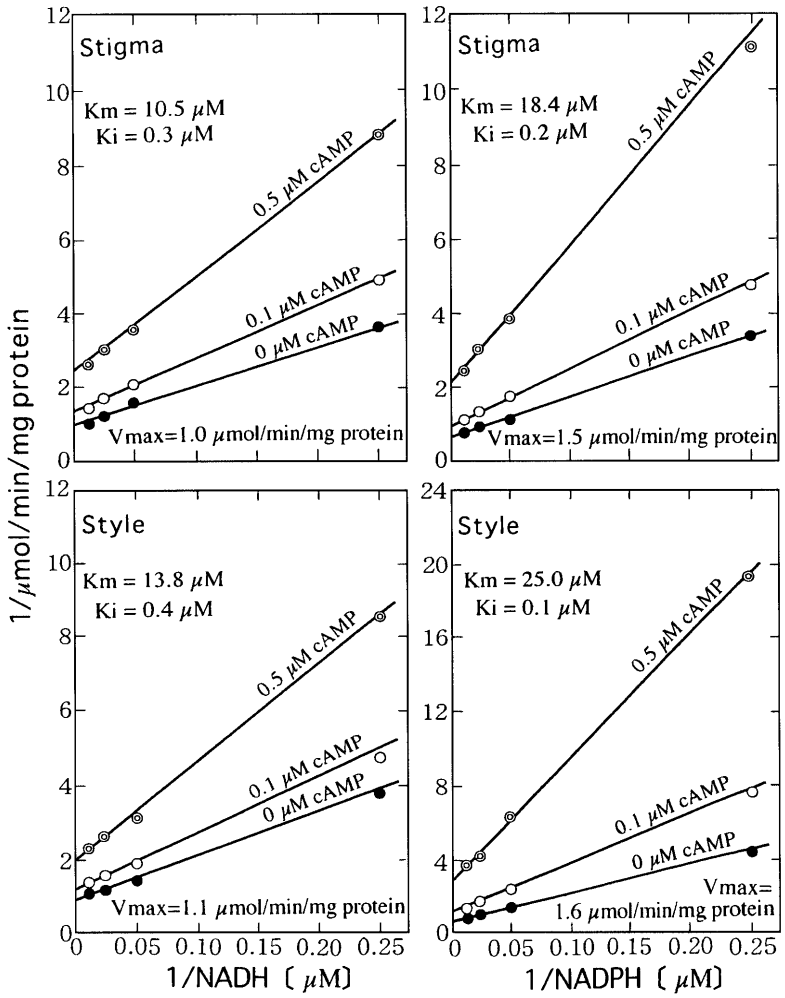

Fig. 1 The double reciprocal plot of NADH- and NADPH-dependent oxidase activity as a function of NADH and NADPH concentration in the membrane fraction from stigmas and styles of *Lilium longiflorum* in the presence and absence of cAMP. A double reciprocal plot was calculated from the data of dose-response to NADH and NADPH for NADH- and NADPH-dependent oxidase.

from stigmas and styles showed almost the same hyperbolic curve either in the presence or absence of cAMP (data not shown). Double-reciprocal plots were made from isotherms of both NADH- and NADPH-dependent oxidases for soluble fractions from stigmas and styles (Fig. 2). NADH gave K_m and V_{max} values for NADH-dependent oxidase in soluble fraction from stigmas of approximately 6.9 μM and 2.3 μmol (mg protein)$^{-1}$ min^{-1}, respectively and NADPH 12.7 μM and 2.7 μmol (mg protein)$^{-1}$ min^{-1}, respectively. The K_m and V_{max} for NADH-dependent oxidase in the soluble fraction from styles were 13.0 μM and 1.1 μmol (mg protein)$^{-1}$ min^{-1}, and those for NADPH-dependent oxidases 6.6 μM and 0.6 μmol (mg protein)$^{-1}$ min^{-1}, respectively.

Tezuka et al. (1997) examined the stress enzymes in the extracts from stigmas and styles of Hinomoto lilies after pollination, and found their activities to be higher after self-incompatible pollination than after cross-compatible pollination. This implies that the elongation of pollen tubes in pistils of Hinomoto lilies after self-incompatible pollination and cross-compatible pollination is modulated by the level of active oxygen species in pistils after pollination.

According to Tezuka et al. (1993), the elongation of pollen tubes in the pistils of Hinomoto lily after self-incompatible pollination is promoted by exogenous cAMP. This suggests that the growth of pollen tubes is also regulated by the level of cAMP in the pistils and that the activities of stress enzymes are inhibited by cAMP. In fact, the activities of NADH- and NADPH-dependent oxidases in the extracts from stigmas and styles of non-pollinated Hinomoto lilies were decreased by 0.1 μM cAMP (Table 1). The decrease in the activities of NADH- and NADPH-dependent oxidases in the extracts from pistils by cAMP was

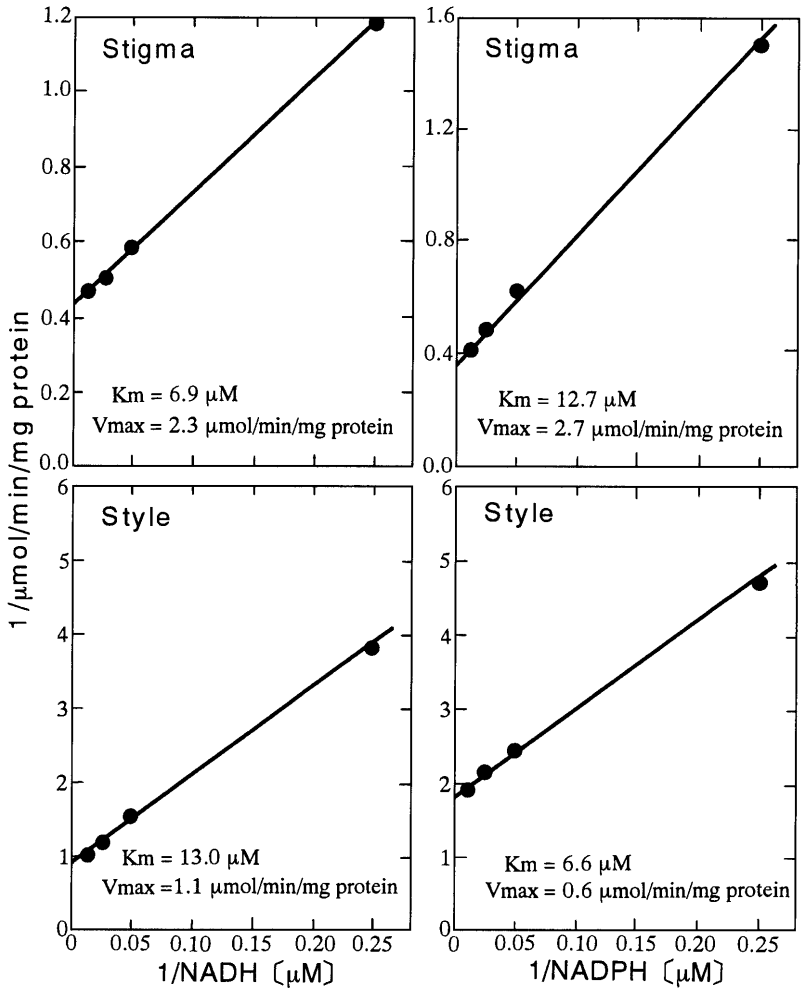

Fig. 2 The double reciprocal plot of NADH- and NADPH-dependent oxidase activity as a function of NADH and NADPH concentration in the soluble fraction from stigmas and styles of *Lilium longiflorum* in the presence and absence of cAMP. A double reciprocal plot was calculated from the data of dose-response to NADH and NADPH for the NADH- and NADPH-dependent oxidase.

similar to that in the extract from leaves (not flower tissues) of Hinomoto lily (data not shown), as was the case in animal samples such as neutrophils (Takenawa et al. 1986). As a rule, the inhibition of NAD(P)H-dependent oxidase activity by cAMP may be possible in both plants and animals.

The activities of other stress enzymes such as xanthine oxidase, SOD, catalase and ascorbate peroxidase in the extracts from stigmas and styles were not decreased by cAMP at 0.01–10 μM (data not shown). Furthermore, the decrease in the activities of NADH- and NADPH-dependent oxidases by cAMP was observed in the membrane fraction (not soluble fraction) from stigmas and styles (Table 1). We suppose that cAMP may regulate the activity of specific enzymes in the O_2^--forming system. In lily pistils, only NADH- and NADPH-dependent oxidases in the membrane fraction may be inhibited by cAMP, and keep the O_2^- level in the pistils of Hinomoto lily low (cf. Tezuka et al. 1993, 1997).

In the absence of cAMP, the K_m for NADH (stigma; 10.5 μM, style; 13.8 μM) for the NADH-dependent oxidase in the membrane fractions from stigmas and styles was smaller than the K_m for NADPH (stigma; 18.4 μM, style; 25.0 μM) for the NADPH-dependent oxidase (Fig. 1). Since the affinity of NADH for the NADH-dependent oxidase was greater than that of NADPH for the NADPH-dependent oxidase, in the membrane fractions. This seems to be opposite to that observed in animal cells. Usually, in most animal cells, the K_m for NADPH for NADPH-dependent oxidase is 25–50 μM and the K_m for NADH for NADH-dependent oxidase 0.5–1.0 mM (Wakeyama et al. 1982). NADH-dependent oxidase is considered to play a more important role than NADPH-dependent oxidase in the membrane fraction of pistils of Hinomoto lily. By

contrast, in the presence of cAMP at 0.1 μM and 0.5 μM the K_i for NADH for the NADH-dependent oxidase in the membrane fraction was greater than the K_i for NADPH for the NADPH-dependent oxidase (Fig. 1). cAMP seems to inhibit the activity of NADPH-dependent oxidase more strongly than that of NADH-dependent oxidase. To our knowledge, it remains unknown whether or not NADH- and NADPH-dependent oxidases are different enzymes and are localized in different sites of the cell membrane. The difference in the K_m and the K_i between NADH- and NADPH-dependent oxidases indicates that NADH-dependent oxidase and NADPH-dependent oxidase may use only NADH and NADPH, respectively. The inhibition by cAMP seems to be noncompetitive with NAD(P)H as shown in Figure 1, since cAMP may alter the conformation of NADH- and NADPH-dependent oxidases and inhibit the enzyme activities. In the soluble fractions, neither NADH- nor NADPH-dependent oxidase was affected by cAMP (data not shown). Thus, cAMP may specifically associate with membrane-bound NADH- and NADPH-dependent oxidases, but not with the soluble enzymes. Because adenylate cyclase (cAMP-forming enzyme) (Rodbell et al. 1971) and NAD(P)H-dependent oxidase (superoxide-forming enzyme) (Wakeyama et al. 1982) are known as membrane-bound enzymes, the activity of NAD(P)H-dependent oxidase seems to be inhibited by cAMP which is produced by adenylate cyclase. The site for O_2^--formation by NADPH oxidase, which is localized to pierce the membrane of cells, is the apoplast-side and the binding site of NADPH as a substrate is the symplast-side (Doke 1996).

Pistils of Hinomoto lilies may use cAMP as a second messenger in the control of the metabolism in cells. Legender et al. (1997) detected significant quantities of cAMP in and around the stigmas, styles, ovaries and anthers of *Lilium* cv. Connecticut. Furthermore, they confirmed the presence of cAMP in the lily flowers by measuring the adenylate cyclase activity in the membrane fraction of the extract from the upper part of a style including the stigma. We also found that the levels of endogenous cAMP in the stigmas and styles of Hinomoto lily after cross-compatible pollination were approximately two-fold higher than those after self-incompatible pollination (unpublished data). Moreover, the elongation of pollen tubes in the pistils of Hinomoto lily with self-incompatible pollination was enhanced by an activator (forskolin) of adenylate cyclase activity and an inhibitor (3-isobutyl-1-methylxanthine) of cyclic nucleotide phosphodiesterase activity, besides exogenous cAMP (Tezuka et al. 1993). These results suggest that Hinomoto lily possesses adenylate cyclase to produce cAMP, but not as a putative component, as in flower organs of Lilium cv. Connecticut (Legender et al. 1997).

In conclusion, the activity of NADH- and NADPH-dependent oxidases, i.e., O_2^--forming enzymes in the membrane fraction but not in the soluble fraction from pistils of Hinomoto lily without pollination was inhibited

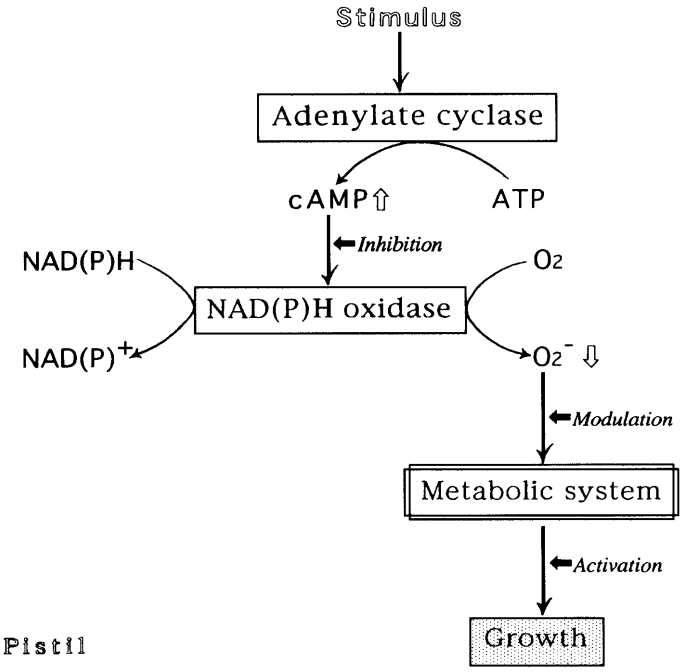

Fig. 3 The schematic representation of a putative model for the relationship between the activity of NAD(P)-dependent oxidase and cAMP in the pistils of *Lilium longiflorum*. ⇧: increase in cAMP, ⇩: decrease in superoxide (O_2^-).

by cAMP. A schematic representation of a putative model for the relationship between the activity of superoxide-forming enzyme and cAMP in the pistils of Hinomoto lily is shown in Figure 3. In Hinomoto lily, cross-compatible and self-incompatible pollination acts in a positive and negative way, respectively, through cAMP in pistils. Adenylate cyclase activated and/or induced by the stimulus through pollination may maintain the high levels of cAMP which plays an important role in the stress signaling for NAD(P)H-dependent oxidase.

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