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Copper and zinc ions induce both stylar RNase inhibition and fruit set in Japanese pear

Taishi Hayashida*, Akihiro Horikawa, Kazuyoshi Nada, Shin Hiratsuka

Graduate School of Bioresources, Mie University, Tsu, Mie 514-8507, Japan

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ABSTRACT

When inhibitory effects on stylar RNase activity were investigated in the Japanese pear (*Pyrus pyrifolia* Nakai), $CuSO_4$ and $ZnSO_4$ were significantly effective, and the inhibition was dose-dependent. Since $MgSO_4$ and $CaCl_2$ did not show any inhibition, the active ions would be Cu^{2+} and Zn^{2+}. Because Cu^{2+} separated clearly from the stylar proteins when protein–$CuSO_4$ mixture was loaded on Sephadex G-10 column chromatography, the cation may not be combined strongly with the protein. The RNase activity of the protein, furthermore, was almost completely recovered when the proteins were isolated from the mixture by $(NH_4)_2SO_4$ precipitation. Copper ions strongly reduced the RNase A activity, moderately RNase S and weakly RNase T_1 at 1 mM, while it did not affect the RNase B. The $CuSO_4$ application at 2 mM induced more than 30% fruit set following self-pollination but Na_2SO_4 and K_2SO_4 did not, suggests that Cu^{2+} causes fruit set through reducing stylar RNase activity.

1. Introduction

Self-incompatibility is a genetically controlled mechanism in flowering plants which prevents inbreeding and promotes out crossing. Since Japanese pear (*Pyrus pyrifolia* Nakai) exhibits S-RNase-based self-incompatibility, artificial pollination is conducted in Japan because fertilization and subsequent seed formation are inevitable for fruit setting and fruit growth. After pollination, furthermore, fruit thinning should be done at fruit:leaf ratio = 1:25 to promote fruit growth. Breeding of self-compatible cultivars using self-compatible 'Osa-Nijisseiki' has been proceeded and several new cultivars released recently in Japan (Washio et al., 2006), but they need much labor for fruit thinning. Therefore, development of cultivars with moderate fruit set and/or suitable chemical thinners would be desirable; only 5% of flowers are necessary for fruit production (Hiratsuka et al., 2002).

In S-RNase-based self-incompatibility systems, the RNase activity is necessary for rejecting incompatible pollen (Huang et al., 1994; Kowyama et al., 1994; Royo et al., 1994; McCubbin et al., 1997; Hiratsuka et al., 2001), and the rejection ability decreases with decreasing S-RNase activity (Qin et al., 2006). Recently, $ZnSO_4$ and $CuSO_4$ were reported to reduce S-RNase activity of tomato, and induce enough fruit set after self-pollination (Kim et al., 2001). Based on this information, a compound "apple plus" containing these salts has been developed by Korean company for overcoming apple self-incompatibility (Chung et al., 2005). According to the patent report, this chemical can reduce S-RNase activity by 60% of control, and induce fruit set about 50% in wild tomato and more than 60% in apple after self-pollination by the treatment 1–2 days before anthesis. This suggested that pear cultivation without pollination and fruit thinning could be established by adjusting concentration and/or treatment time of the "apple plus". However, we could not obtain the satisfactory effect of the chemical in the pear (Hiratsuka et al., 2009), and physiological mechanism on RNase inhibition is still unclear.

In this study, therefore, we aimed to screen the adequate metal salt for inhibiting stylar RNase of the pear first, and then to elucidate possible mechanism of the salt on RNase inhibition, and finally to confirm the effects on fruit set in the pear.

2. Materials and methods

2.1. Plant materials

The trees of Japanese pear (*P. pyrifolia* Nakai) 'Kosui' were used at the experimental farm of Mie University, Tsu, Mie, Japan. For stylar protein extraction, styles were collected from the flowers just before anthesis and stored in liquid nitrogen until use.

2.2. Stylar protein extraction

All procedures were done below 4 °C. Proteins were extracted by the partially modified method of Hiratsuka et al. (1995). Briefly, styles were ground in liquid nitrogen using a mortar and pestle, and

* Corresponding author. Tel.: +81 592321211; fax: +81 592319540.
E-mail address: 511d303@m.mie-u.ac.jp (T. Hayashida).

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the extraction buffer [50 mM Tris–HCl buffer (pH 8.4) containing 150 mM NaCl, 10 mM L-cysteine, 1 mM ascorbic acid, 1 mM $CaCl_2$, 10 mM KCl, 1 mM Na_2-EDTA and Polyclar-AT (0.3 g/gfw)] was added immediately. After stirring the homogenate, it was kept for 30 min, centrifuged at 15,000 × *g* for 10 min and then the supernatant was passed through a Sephadex G-25 column to remove polyphenols. The protein fractions pooled were saturated with ammonium sulfate at a final concentration of 100%. After leaving for 30 min, it was centrifuged again at 15,000 × *g* for 10 min to precipitate the proteins. The protein pellet was dissolved with a small amount of the Tris–HCl buffer (pH 8.4), dialyzed against the same buffer overnight, and stored at −30 °C until use. Protein concentration was determined by the method of Bradford (1976).

2.3. Screening of metal salts as the stylar RNase inhibitor

To test the inhibitory effect on pear RNase activity, following chemicals were used: $CaCl_2$, $ZnSO_4$, $MgSO_4$, $CuSO_4$, $FeSO_4$ and $Pb(NO_3)_2$. The $CaCl_2$ was purchased from Wako Pure Chemicals Ltd. (Osaka, Japan) and the others from Nakarai Chemicals Ltd. (Kyoto, Japan). The concentration of metal salts used was 1 mM because S-RNase of *Lycopersicon peruvianum* was inhibited significantly at this concentration (Kim et al., 2001). RNase activity was determined according to the method of Brown and Ho (1986). The reaction mixture contained 50 μg of proteins, 500 μL of 4 g L^{-1} torula yeast RNA (Sigma, USA), 10 mM KCl and each metal salt. The total volume was adjusted to 750 μL with 50 mM Tris–HCl buffer (pH 8.4). After incubation for 30 min at 55 °C, 250 μL of 20% trichloroacetic acid was added to stop the reaction. The mixture was stirred, centrifuged at 15,000 × *g* for 10 min, and then the supernatant was diluted 100-fold with distilled water and measured OD260 using a UV-1800 spectrophotometer (Shimadzu, Japan). As a control, Tris–HCl buffer was added instead of metal solution.

Since $CuSO_4$ and $ZnSO_4$ showed strong inhibition, the inhibitory effect of $CuSO_4$ was studied at various concentrations, 0.25–2 mM. All experiments were carried out 3 times.

2.4. Inhibitory effect of $CuSO_4$ on several RNases

Inhibitory function of $CuSO_4$ on RNase A, RNase S, RNase B, and RNase T_1 was investigated; about 20 U of each enzyme was assayed as described above under presence or absence of 1 mM $CuSO_4$. RNase A was purchased from Wako Pure Chemicals Ltd. (Osaka, Japan), and RNase S, B and T_1 were from Sigma Chemical Co. Ltd. (USA). Experiment was repeated at least 3 times. Results were expressed as % of control.

2.5. Sephadex G-10 column chromatography of protein–copper mixture

One milliliter of 50 mM Tris–HCl buffer (pH 8.4) containing 5 mM $CuSO_4$ and 2.5 mg stylar proteins was loaded on a Sephadex G-10 gel column (17 mm width × 250 mm length) equilibrated with 50 mM Tris–HCl buffer. The elution was fractionated in 2 mL each, then protein and copper ion concentrations of each fraction were determined by the method of Bradford (1976) and atomic absorption spectrophotometer (AA-6200; Shimadzu, Japan), respectively.

2.6. RNase activity of isolated proteins by ammonium sulphate from $CuSO_4$–protein mixture

After 50 μg of stylar proteins were mixed with 2 mM $CuSO_4$ solution and kept for 5 min, the proteins were salted out by $(NH_4)_2SO_4$ at a final concentration of 70%. The proteins were gathered by centrifuging at 15,000 × *g* for 10 min and dissolved with small amount of 50 mM Tris–HCl buffer (pH8.4). Then, RNase activity of the proteins was determined as described above. As a control, the same amount of buffer was used instead of $CuSO_4$ solution. Experiment was repeated 3 times.

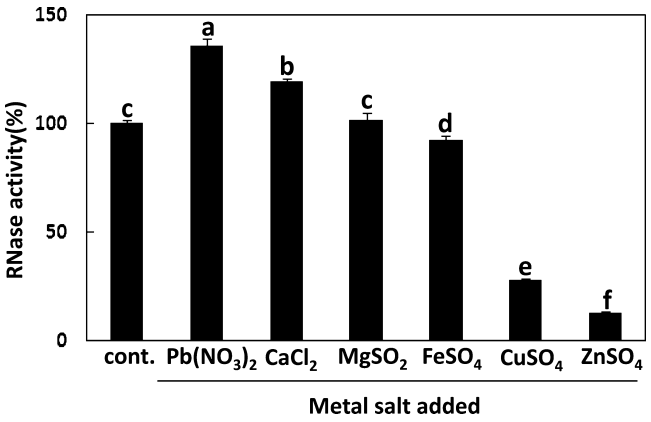

Fig. 1. Effect of several metal salts on stylar RNase activity of the pear. Each treatment contained 50 μg protein and 1 mM metal salt. Control contained buffer only. Each value is expressed as % of control. Vertical bars indicate SE. Different letters indicate significance at $P < 0.05$ by Duncan's multiple range test.

2.7. Fruit set induction by $CuSO_4$ application in the pear

The 2 mM $CuSO_4$ solution contained 0.1% Tween-20 as a spreading agent was sprayed on the flower buds of 'Kosui' 10 days before anthesis, and the clusters were covered with paper bags to prevent contamination. At the day of anthesis, self-pollination was carried out and bagged again, and fruit set was checked 2, 3 and 4 weeks after pollination. As a control, 0.1% Tween-20 solution was used. Seventy flowers were used for treatment and control, respectively.

To know whether copper ion induces fruit set, the effects of 2 mM Na_2SO_4 and K_2SO_4 were also sprayed, respectively.

2.8. Statistical analysis

Data were calculated using Microsoft Excel (ver. 12.0.6425.1000) and standard errors were suggested at each data point. The significance between the data was analyzed by Duncan's multiple range test. In the fruit set experiment of Fig. 6, the significance of regression curve in $CuSO_4$ treatment was analyzed by R 2.15.1 software (R Development Core Team, 2010), and significance of each data by Fisher's exact test. The column chromatography was conducted 3 times and typical profile was shown in this manuscript. Fruit set experiment was carried out over 2 years and data in 2009 were presented here.

3. Results

3.1. Screening of metal salts as the stylar RNase inhibitor

The inhibitory effects on stylar RNase activity were examined in the pear *in vitro* using several metal salts (Fig. 1). The $Pb(NO_3)_2$, $CaCl_2$ and $MgSO_4$ did not show any inhibition but former two chemicals somewhat promoted the activity. Strong inhibition was observed in $CuSO_4$ and $ZnSO_4$ addition and weak inhibition in $FeSO_4$; $CuSO_4$ and $ZnSO_4$ suppressed the activity more than one-third of control, respectively. When the action of $CuSO_4$ was tested at various concentrations, from 0.25 to 2 mM, the inhibition was dose-dependent (Fig. 2).