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Effect of SS-toxin, a metabolite of *Stemphylium solani*, on H^+-ATPase activity and standard redox system in plasma membranes from seedlings leaves of garlic (*Allium sativum*)

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Abstract Leaf blight of garlic (*Allium sativum*) is a severe disease in garlic-growing regions. SS-toxin is a newly described non-proteinaceous toxin produced by the phytopathogenic fungus, *Stemphylium solani,* the cause of garlic leaf blight. In this study, the effects of SS-toxin on the H^+-ATPase activity and standard redox activity in the plasma membranes isolated by aqueous polymer two-phase partitioning from garlic seedling leaves were studied in vitro. The H^+-ATPase activity, NADH oxidation rate and $Fe(CN)_6^{3-}$ reduction rate of the plasma membranes isolated from susceptible and resistant cultivars were all inhibited in a dose-dependent manner. Our results suggest that, under in vitro conditions, the plasma membrane H^+-ATPase and standard redox system can be both the cellular targets of SS-toxin.

Keywords H^+-ATPase · $Fe(CN)_6^{3-}$ reduction rate · Garlic · NADH oxidation rate · Toxin

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Introduction

The phytopathogenic fungus, *Stemphylium solani,* causes leaf blight of garlic (*Allium sativum*), a serious disease in vegetable-growing areas of China (Zheng et al. 2008a). The symptoms of the disease initially consist of small white spots enlarging to elongated purple necrotic lesions along the vein followed by blighting of the leaf tips. The pathogen produces a non-host-specific toxin, SS-toxin, which is capable of causing symptoms normally associated with leaf blight (Zheng et al. 2009). However, the chemical structure and precise mode of action of SS-toxin has not been elucidated. The primary site of action of some phytotoxins produced by phytopathogenic fungi is thought to be the plasma membrane (Marre 1980). Our preliminary studies indicated that *S. solani* toxin might act to induce the flaccidity of plant tissues as a result of interference with cellular membrane systems, and the first induced changes appeared on plasma membranes of garlic leaf cells.

The H^+-ATPase, which acts as a major transporter by pumping protons out of the cell, is a key enzyme

in the plasma membrane for plants. It plays an important role in cell wall formation, nutrient uptake, volume regulation and stomatal guard cell movements, and its activity is likely to be regulated by various factors, including light, plant hormones and phytotoxins (Serrano 1990). This enzyme has been reported to be a target for several fungal toxins and elicitors, which either inhibit or stimulate its activity (Ballio 1991). Blein et al. (1988) reported that the hydrolytic activity of the H^+-ATPase is inhibited by *Cercospora beticola* toxin in microsomal vesicles from maize roots. The phytotoxin fusicoccin, a major metabolite of *Fusicoccum amygdale*, promotes growth and affects ion fluxes across the plasma membranes of oat roots (Lanfermeijer and Prins 1994). These responses are generally involved in stimulation of the plasma membrane H^+-ATPase (Marre 1979). However, effects of phytotoxins produced by species of *Stemphylium* on the H^+-ATPase activities have not yet been studied.

Generation of proton electrochemical potential gradient is also dependent on the plasma membrane redox system which is an electron transfer chain including electron donor oxidases, electron vectors and electron acceptor reductase, et al. (Barr 1991). Redox activity could play an important role in preventing the inactivation of transport proteins. One of the functions of the plasma membrane redox system might be to keep the sulfhydryl portion of membrane transport proteins in a reduced state, thus preventing their inactivation by oxidation (Kochian and Lucas 1991). The involvement of NADH oxidation in the generation of active oxygen species and H_2O_2 has been established in different plant systems (Elstner 1982). Plasma membrane-bound NADH oxidase had been found to generate superoxide radicals upon infection of plant hosts with phytopathogenic fungi or phytotoxin treatments (Doke 1985; Bugiani et al. 1998).

In the present study, we measured the in vitro activity of garlic leaf plasma membrane H^+-ATPase and standard redox system in the presence of SS-toxin. Two garlic cultivars, susceptible cv. Changbanpo and resistant cv. Qingganruanye were compared for their response to SS-toxin over a wide range of concentrations. In artificial inoculation experiments, Qingganruanye exhibited only a few small white spots 3 d after inoculation, and was much more resistant to *S. solani*, compared to the susceptible Changbanpo which showed purple necrotic lesions (Zheng et al. 2009). By using plasma membranes alone, we attempted to isolate the effects to activities associated with these membranes, precluding the possibly confounding action of the toxin on other cellular targets.

Materials and methods

Production and purification of toxin

A virulent isolate (DY-5) of *S. solani* used for toxin production and culture filtrate was obtained in Zheng et al. (2009). The filtrate was extracted three times with half volume ethyl acetate. The ethyl acetate extracts were combined, and evaporated to dryness with a Model RE52 rotary evaporator (Yarong, Shanghai, China) at 50°C. A yellow-brown residue was obtained and dissolved in heated methanol. The SS-toxin was purified following the method of Zheng et al. (2008b). The residue was first subjected to thin-layer chromatography (TLC). Preparative TLC plates coated with a GF-254 fluorescent silica gel (5×20 cm, Qingdao, China) were used and separately developed in a solvent system containing ethyl acetate/sherwood oil/methanol (4:1:0.35, *v*/*v*/*v*). Bands on the TLC plates were marked under ultraviolet (UV) light at 254 nm. Target bands were carefully scraped off the plates, dissolved in ethyl-acetate, filtered through Whatman no. 1 filter paper, and centrifuged at 8,000 rpm for 10 min. The ethyl acetate was dried with a rotary evaporator at 50°C. The toxin residue obtained from TLC plates was dissolved in methanol, filtered through 0.22 μm filters (Luqiaosijia Company, Taizhou, China) to remove insoluble materials, and then purified with preparative liquid chromatography (LC). Preparative LC was performed on a PrepLC 500A system (Waters, Milford, USA) using a 5.7×30 cm column packed with 15–20 μm Vydac C_{18} (Vydac, Hesperia, USA), a methanol/water (40:60, *v*/*v*) solvent system, a flow rate of 1 ml min^{-1} and 215-nm detection. The purified SS-toxin was dried with a rotary evaporator at 50°C, and then stored at 4°C until used.

Preparation of plasma membrane

Plasma membrane of garlic (*Allium sativum*) was prepared using a two-phase partition system following

the procedure of Sandelius and Morre (1990) with minor modifications. Garlic leaves of susceptible cv. Changbanpo and resistant cv. Qingganruanye (20-d-old) were used in this study. Leaves (50 g) of each cultivar were homogenized with a blender in 100 ml of grinding medium that consisted of 0.25 M sucrose, 3 mM EDTA, 2.5 mM DTT, 0.6% (*w*/*v*) PVP, 2.5 mM PMSF and 25 mM Tris-Mes (pH 7.8). The homogenates were filtered through four layers of cheesecloth and centrifuged at 13,000 g for 15 min. The supernatants were then centrifuged at 80,000 g for 30 min to obtain microsomal pellets that were suspended in buffer containing 2 mM Tris-Mes (pH 7.8), 0.25 M sucrose, 0.1 mM DTT, and 5 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.8). The microsomal fractions were then added to a polymer phase system with a final weight of 27.0 g and a final concentration of 6.2% (*w*/*w*) Dextran T-500, 6.2% (*w*/*w*) PEG 3350, 0.25 mM sucrose and 5 mM KH_2PO_4-K_2HPO_4 buffer (pH 7.8). The two-phase partitioning was repeated three times. The final upper phases were diluted 5-fold with 0.25 mM sucrose and 5 mM KH_2PO_4-K_2HPO_4 buffer (pH 6.8), and centrifuged at 100,000 g for 30 min. The resulting pellets were re-suspended in a wash solution containing 0.25 mM sucrose and 5 mM KH_2PO_4-K_2HPO_4 buffer (pH 6.5), quickly frozen in liquid N_2, and stored at −80°C. All steps of plasma membrane preparation were carried out at 4°C.

The purity of prepared plasma membranes was estimated from measurements of enzymatic activities of plasma membranes and other organelles. After separate additions of specific inhibitors vanadate (Na_3VO_4), nitrate (KNO_3), azide (NaN_3) or molybdate (Na_2MoO_4), the activity of H^+-ATPase was assayed by measuring released inorganic phosphorus (Pi) from ATP at pH 6.5 as described by Widell and Larsson (1990). The H^+-ATPases of plasma membrane, tonoplast and mitochondria belong to P-, V- and F-types, characterized by inhibition by vanadate, nitrate and azide, respectively, and non-specific phosphatases identified by molybdate inhibition (Larsson et al. 1987).

In vitro treatment

The plasma membranes of both cultivars were diluted to a final protein concentration of 0.5 μg ml^{-1} and incubated at 25°C for 30 min with toxin solution (0.1, 1, 10, 50, 100 or 200 μg ml^{-1}) in the presence of 0.02% (*v*/*v*) Triton X-100 following the procedure of Batoko et al. (1998) with minor modifications.

Measurement of H^+-ATPase activity

The H^+-ATPase activity of pre-treated plasma membrane was performed according to the method of Chen et al. (2004) with minor modifications. Membrane proteins (10 μg) were added to 0.5 ml of reaction medium containing 3 mM ATP, 3 mM $MgSO_4$, 50 mM KCl, 1 mM NaN_3, 50 mM KNO_3, 0.1 mM Na_2MoO_4, 25 mM Tris-Mes (pH 6.5) and 0.02% Triton X-100. After 30 min incubation at 37°C, the reaction was stopped by the addition of 10% (*w*/*v*) trichloroacetic acid. The H^+-ATPase activity was determined by measuring the released inorganic phosphorus (Pi) at 660 nm with a Mapada UV-1600PC spectrophotometer (Mapada, Shanghai, China).

Measurement of NADH oxidation and ferricyanide reduction rate

NADH oxidation and $Fe(CN)_6^{3-}$ reduction rates of pre-treated plasma membrane were measured by the method of Gong et al. (2003) with minor modifications. The reaction solution contained 10 mM Tris-Mes (pH 8.0), 0.25 M sucrose, 0.25 mM NADH, 1 mM $Fe(CN)_6^{3-}$, 0.02% Triton X-100 and 0.1 mM KCN. The reaction was started by adding 10 μg plasma membrane protein. The NADH oxidation rate was measured at 340 nm with a Mapada UV-1600PC spectrophotometer and calculated using the extinction coefficient of 6.23 mM^{-1} cm^{-1}. The $Fe(CN)_6^{3-}$ reduction rate was assayed spectrophotometrically at 420 nm, and the extinction coefficient of 1.00 mM^{-1} cm^{-1} was used in calculations of reduction rate.

Protein determination

Protein content was determined by the method of Bradford (1976) using bovine serum albumin as a standard. The dye Coomassie blue G250, was used to bind to plasma membrane proteins. The binding of the dye to protein caused a shift in the absorption maximum from 465 to 595 nm, and absorption at 595 nm was measured with a Mapada UV-1600PC spectrophotometer.

Statistical analyses

All experiments were carried out with three replicates. Analysis of variance was performed using a two factor completely randomized design. The data were subjected to analysis of variance (ANOVA), and when significant treatment differences were found ($P \leq 0.05$), means were compared by the test of least significant difference (LSD, $P=0.05$).

Results

The plasma membranes prepared by aqueous polymer two-phase partitioning are predominantly vesicles that are sealed right-side-out (apoplastic side out) (Larsson et al. 1987, 1994). Since the H^+-ATPase is located on the cytoplasmic surface of the plasma membrane, the right-side-out vesicles should be inverted into the inside-out (cytoplasmic side out) orientation for further H^+-ATPase-related study (Palmgren et al. 1990). Thus, we tested the efficiency of a detergent, Triton X-100 for this purpose. In the presence of Triton X-100, H^+-ATPase activity of plasma membrane from leaves of cv. Changbanpo and cv. Qingganruanye was increased from 2.2 to 10.6 μmol Pi mg^{-1} protein min^{-1} and from 3.0 to 11.1 μmol Pi mg^{-1} protein min^{-1}, respectively (Table 1). Moreover, there was no difference in composition of the plasma membranes, whether in the absence or presence of Triton X-100, isolated from leaves of these two garlic cultivars. The plasma membrane enriched preparations showed no significant contamination by other membrane systems, as confirmed by assays tested in the presence of selective inhibitors. Total H^+-ATPase activities were more than 70% inhibited by vanadate in both cultivars, whereas the activities were affected to a minor extent by nitrate, azide or molybdate (Table 1).

The H^+-ATPase activities of plasma membranes, isolated from both cultivars, were inhibited by SS-toxin in a dose-dependent manner (Fig. 1a). When the concentration of toxin was increased from 0.1 up to 200 μg ml^{-1}, the relative activity of H^+-ATPase in plasma membrane extracts of resistant cv. Qingganruanye decreased from 92.1% to 56.4% and for susceptible cv. Changbanpo, from 93.4% to 41.4%.

The NADH oxidation and $Fe(CN)_6^{3-}$ reduction rates of plasma membranes from both cultivars were inhibited by SS-toxin at all tested concentrations ranging from 0.1 to 200 μg ml^{-1}, and the inhibition appeared to be proportional to the concentration of toxin (Fig. 1b, c). Using ferricyanide as an electron receptor, the relative NADH oxidation rate in the resistant or susceptible cultivar was reduced to 45.8% or 43.1%, respectively, after treatment with SS-toxin. When NADH was used as an electron donor, the relative $Fe(CN)_6^{3-}$ reduction rate in the resistant or the susceptible cultivar was decreased to 45.4% and 18.9%, respectively.

Discussion

In this study, we tested the effect of various concentrations of SS-toxin on plasma membranes obtained from garlic leaves of two cultivars using an in vitro system. In general, in vitro inhibition can be observed easily within minutes, whereas in vivo effects might

Table 1 Effects of inhibitors on H^+-ATPase activities in plasma membranes isolated from garlic leaves (20-d-old) of two cultivars, Changbanpo and Qingganruanye

Inhibitor (mM)	Triton X-100	H^+-ATPase activity (μmol Pi mg^{-1} protein min^{-1})[a]	
		Changbanpo	Qingganruanye
None	None	2.2±0.2 g	3.0±0.5 f
None	0.02%	10.6±0.4 bc	11.1±0.4 a
KNO_3 (50)	0.02%	9.8±0.1 d	10.7±0.1 abc
NaN_3 (1.0)	0.02%	9.3±0.2 e	9.8±0.2 d
Na_3VO_4 (1.0)	0.02%	2.6±0.3 fg	2.9±0.1 f
Na_2MoO_4 (0.1)	0.02%	10.4±0.3 c	11.0±0.3 ab

[a] Analysis of variance was performed using a two factor completely randomized design. Means (and their standard deviations) followed by a letter in common are not significantly different at $P<0.05$ in a test of Least Significant Difference

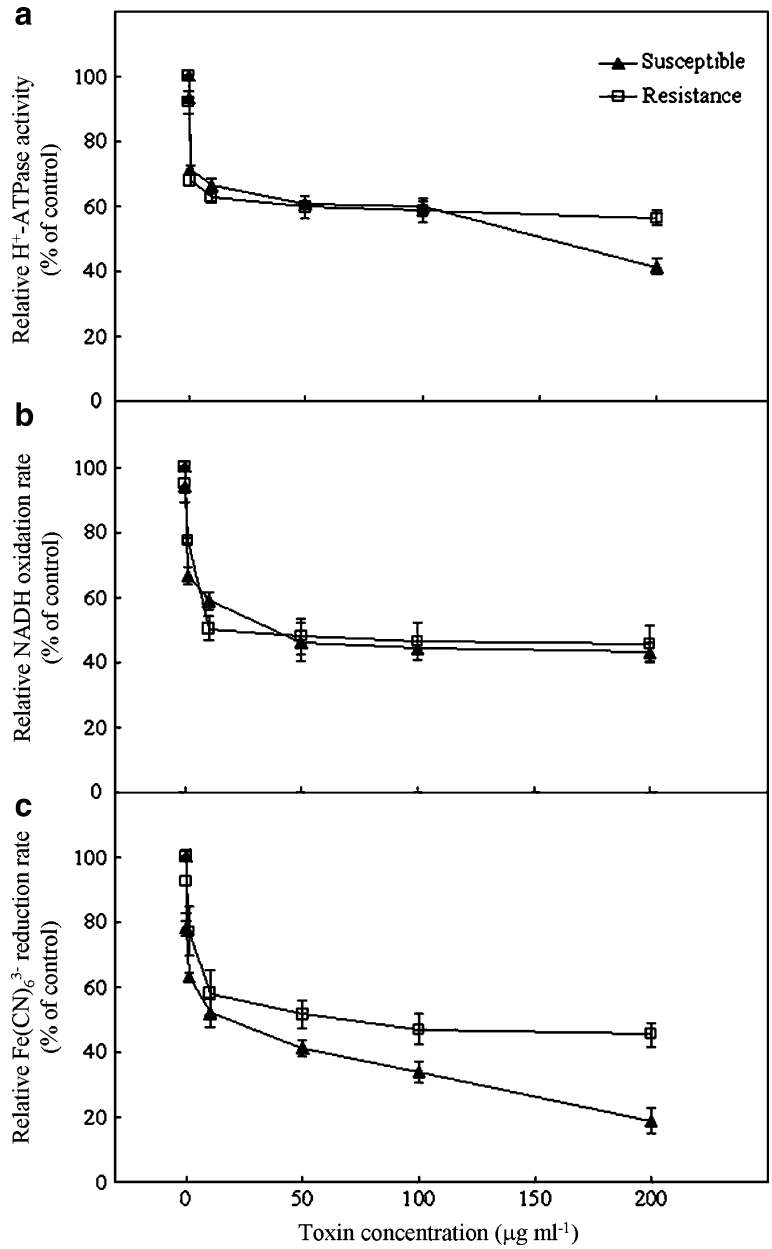

Fig. 1 Effect of various concentrations of SS-toxin on **a** H^+-ATPase activity, **b** NADH oxidation rate and **c** $Fe(CN)_6^{3-}$ reduction rate in plasma membranes isolated from garlic leaves (20-d-old) of two cultivars, Changbanpo and Qinggan-ruanye. Values are expressed relatively as a mean percentage of the control treatment (9.6±0.3 and 10.1±0.2 μmol Pi mg^{-1} protein min^{-1} for H^+-ATPase activity; 49.5±5.0 and 64.0±8.8 nmol Pi mg^{-1} protein min^{-1} for NADH oxidation rate, 122.6±13.0 and 144.0± 11.4 nmol Pi mg^{-1} protein min^{-1} for $Fe(CN)_6^{3-}$ reduction rate)

require several hours (Batoko et al. 1998). Insight into the mechanism of action of phytotoxins can be provided by this system allowing the effects of phytotoxins on membrane-associated activities to be studied in the absence of other cell components, precluding the possibly confounding action of the toxin on other cellular targets. In preliminary work, visible symptoms on the susceptible cultivar (Changbanpo) could be observed after 72 h of incubation with SS-toxin at concentration above 11 μg ml^{-1}, while a minimum toxin concentration of 44 μg ml^{-1} was needed to cause symptoms on the resistant cultivar (Qingganruanye) (Data not shown). Hence, the SS-toxin concentrations of 0.1~200 μg ml^{-1} covering a wide response range, were used to test the effects on membrane-associated activities.

In leaf cells of the susceptible garlic cultivar, the earliest SS-toxin-induced ultrastructural changes were detected 2 h after toxin treatment of the plasma membranes, and structural damage of mitochondria,

chloroplasts and nuclear membranes was also found with prolonged toxin exposure. These cellular components in the resistant cultivar were disrupted at a much slower rate, and with higher threshhold concentrations (Data not shown).

Although the toxin reduced the activity of the H^+-ATPase of the two cultivars, complete inhibition was not observed. The inhibitory action of phytotoxins affecting H^+-ATPase could result from either a direct interaction of the toxin with the enzyme or through its interaction with the immediate lipid environment, which has been reported to be important for activity (Serrano et al. 1988). These data appeared to be compatible with the hypothesis that inhibitory action was not due to a direct effect on the enzyme. This is consistent with the observation of a non-specific membrane-disrupting effect caused by prehelminthosporol produced by the fungal pathogen, *Bipolaris sorokiniana* (Olbe et al. 1995), which is another member of the Pleosporaceae. Furthermore, we found that the H^+-ATPase activity of susceptible leaf plasma membrane was inhibited more significantly than that of the resistant cultivar by SS-toxin at 200 μg ml^{-1}. Perhaps at this higher concentration of SS-toxin, the vesicles in plasma membranes of the susceptible cultivar became more leaky and hence exposed more active sites to the toxin.

Standard redox systems, which can reduce extracellular electron acceptors, exist widely in plasma membranes of plants. The ion Fe^{3+} is reduced to Fe^{2+} during electron transfer along the redox system, and then absorbed by the plant (Rubinstein and Luster 1993). The results of the present study indicated that a standard redox system also exists in the plasma membrane from garlic leaves. Increased NADH-dependent ferricyanide reduction following the treatment of host plasma membranes with elicitor or other factors suggested that plasma membrane redox activity might result in the production of O_2^- anions, hydrogen peroxide, and other reactive molecules. The increased active oxygen species are potentially involved in the plant defence response against pathogens (Vera-Estrella et al. 1994). However, Bugiani et al. (1998) reported that a partially purified SV-toxin from *S. vesicarium* can induce an increase in the activity of plasma membrane NADH/ferricyaide reductase and an increase in oxygen uptake in susceptible pear leaf tissues. A possible explanation is that SV-toxin only induced the production of low-level active oxygen species and this weak defence response failed to prevent fungal infection. With SS-toxin, we observed that the plasma membrane NADH oxidation and $Fe(CN)_6^{3-}$ reduction rates in susceptible garlic leaves were decreased by SS-toxin. Despite stimulation of NADH oxidation and although $Fe(CN)_6^{3-}$ reduction rate in both resistant and susceptible cultivars was not found after treatment with any of the SS-toxin concentrations tested, the resistant cultivar had a relatively larger $Fe(CN)_6^{3-}$ reduction rate than the susceptible one. Therefore, the resistance of garlic cultivars seems related to the plasma membrane-localized standard redox system. Surprisingly, the responses of the two cultivars differed between the concentrations, 1 and 10 μg ml^{-1}: at 1 μg ml^{-1}, the resistant cultivar had a significantly higher oxidation rate, whereas at 10 μg ml^{-1} the resistant cultivar had the higher activity (Fig. 1b). The reason for this is not known.

In conclusion, the plasma membrane H^+-ATPase and standard redox system in garlic are cellular targets of SS-toxin. To our knowledge, this is the first description of the effects on plant plasma membranes of a phytotoxin produced by the fungus *S. solani*.

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