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Involvement of nitric oxide (NO) in signal transduction of stomatal opening

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Introduction

The stomatal aperture responds to a range of stimuli including light, humidity, CO_2, growth regulators and air pollutants. Many papers have conclusively shown that complex signal transduction pathways in guard cells are involved in the regulation of stomatal movements (Schroeder et al 2001; Zeiger 1990). However, the molecular mechanisms for sensing this variety of stimuli has not yet been revealed. Recent papers have suggested that hydrogen peroxide (H_2O_2) is involved in abscisic acid-induced stomatal closing (Pei et al 2000). H_2O_2 is a reactive oxygen species (ROS) that can diffuse across lipid bilayers, thereby functioning as an intra- and intercellular signaling molecule in plants and animals (Corpas et al 2001; Finkel 1998). In addition to ROS, NO has attracted considerable interest as another diffusable signaling molecule in animal cells. NO is produced within animal cells and exhibits diverse physiological functions including signal transduction, enzyme regulation and immune response (Knowles and Moncada 1994). It is now evident that NO plays essential roles in animal physiology. Although there is an increasing number of reports indicating that NO could also participate in many physiological responses of plants: pathogen response, programmed cell death and growth (Delledonne et al 1998; Durner and Klessig 1999), our understanding of NO in plants is still very limited. Here we demonstrate that NO is involved in the signal transduction mechanisms for stomatal opening.

Materials and methods

Fully expanded leaves (the third, forth and fifth leaves) of *Vicia faba* L. were used for measurements of stomatal aperture. Epidermal peels were carefully detached from the abaxial surface of the leaves in a medium which contained 30 mM KCl, 10 mM MES-KOH (pH 6.15) and 1 mM EGTA. The peels were treated with the medium which contained 10 μM fusicoccin (FC, Sigma-Aldrich, USA), 5 mM *S*-nitroso-*N*-acethyl-penisilamine (SNAP, Dojindo, Japan), 1 mM nitrite or ethanol extract of 5 mM Viagra® (sildenafil citrate, Pfizer, USA) under vacuum for 1 min and then incubated for 20 min under a room light. After the incubation, 10 μM H_2O_2 was added to the peels on a slide glass. Stomatal apertures were determined from measurements of 10-30 digitised

images of stomata using a microscope Microphoto-FXA (Nikon, Japan) connected to a digital image camera (Coolpix 990, Nikon, Japan). Image processing was handled with a NIH image software.

Results and Discussion

NO-induced stomatal opening

Figure 1 shows changes in stomatal apertures of abaxial epidermis peels of *Vicia faba* leaves. We used fusicoccin (FC), a fungal phytotoxin, as a positive control for induction of stomatal opening because FC provides full opening condition through activation of the plasma membrane H^+-ATPase in guard cells. After the treatment of peels with FC, stomatal apertures were increased in contrast with control (Fig. 1a). Similarly, the NO donor SNAP significantly increased stomatal apertures by 35 % more than the control. Stomatal aperture was also increased by a treatment of nitrite (NO_2^-) which can be converted to NO by the activity of endogenous nitrate reductase (NR) (Yamasaki 2000; Yamasaki and Sakihama 2000; Yamasaki et al 1999). The nitrite-induced stomatal open-ing was proportional to nitrite concentration and saturated at 1 mM nitrite (Fig. 1b). The effects of SNAP and nitrite treatments were observed 20 min after the treatments (Fig. 2). These results suggest that NO induces stomatal opening.

Involvement of Viagra-sensitive signaling pathway in stomatal closing

Viagra® (sildenafil citrate), which inhibits cyclic GMP (cGMP) degradation in the NO signaling pathway of humans by inhibiting the activity of phosphodiestrease 5 (Terrett et al 1996), significantly suppressed the H_2O_2-induced stomatal closing (Fig. 3). It has

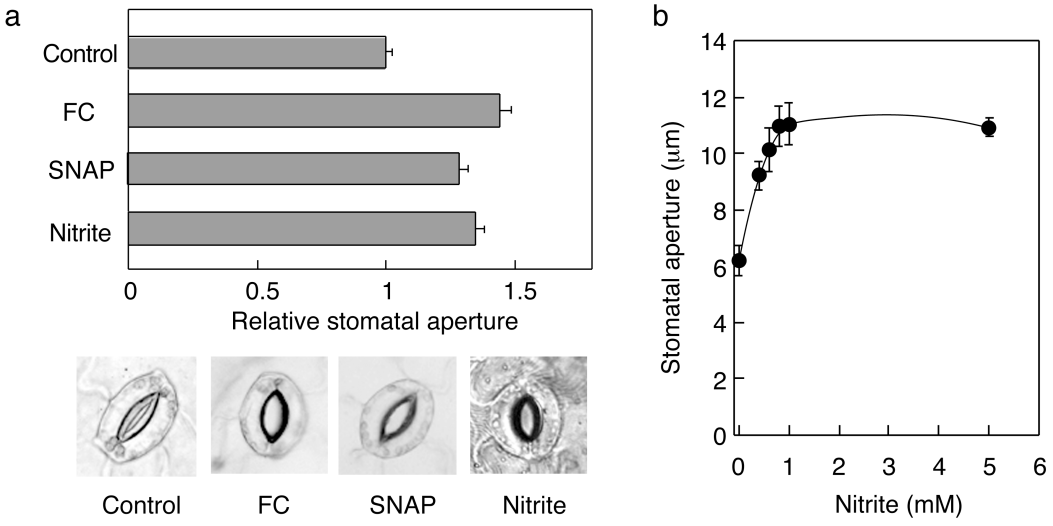

Figure 1. Induction of stomatal opening. a, Increase in stomatal aperture by fusicoccin (FC) and NO donors. b, Effect of nitrite concentration on stomatal opening. Values are means ± SE; n = 10-30. Scale bar: 20 μm.

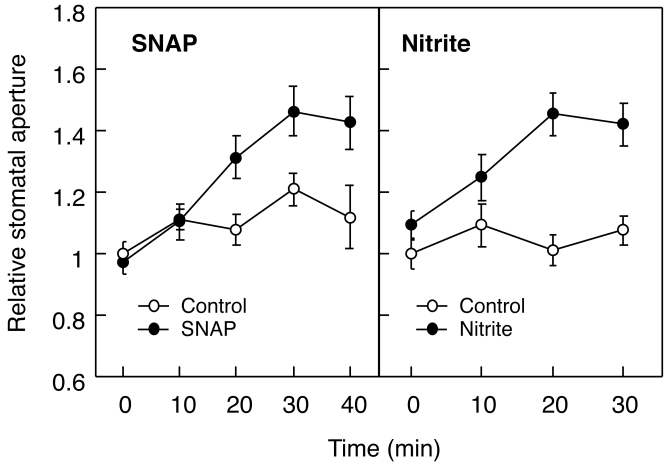

Figure 2. Time courses of stomatal opening in response to SNAP and nitrite. Values are means ± SE; n = 10-30.

been reported that stomatal opening is induced by cGMP in a concentration-dependent manner (Pharmawati et al 1998), agreeing with the results in Fig. 3. These results suggest that an NO-dependent Viagra-sensitive signaling pathway is involved in the stomatal movements. Although active oxygen species are considered to be the signal molecules in the regulatory mechanisms for stomatal movements, our results suggest that active nitrogen species also have to be taken into the consideration.

Source of NO in guard cells

In animal cells, NO is produced by NO synthase (NOS, EC 1.14.13.39) through the enzymatic conversion of L-arginine to L-citrulline. Many studies have suggested the presence of NOS-like activity in plant cells. However, no plant NOS gene homologous to the mammalian one has been cloned to date. Recently, we have reported that NR, a key enzyme of nitrate assimilation in plants, produces NO by the reduction of nitrite (Yamasaki and Sakihama 2000; Yamasaki et al 1999). Although a consensus on the intracellular source of NO in plants has not been reached, nitrite-dependent stomatal opening (Figs. 1 and 2) implies that NR would be responsible for NO production in signal transduction system of guard cells.

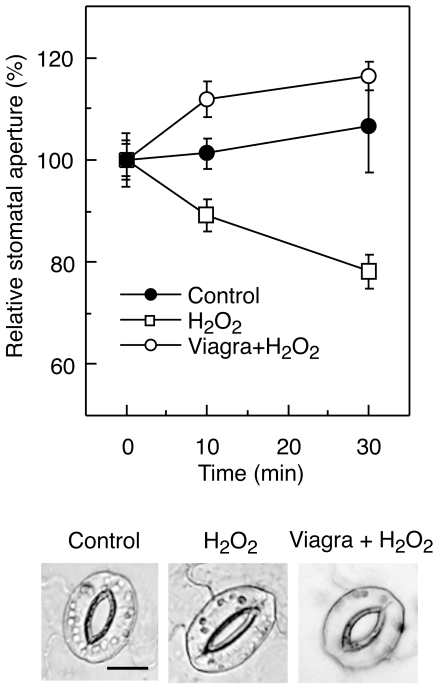

Figure 3. Suppression of H_2O_2-induced stomatal closing by Viagra. Values are means ± SE; n = 10-30. Scale bar: 20 μm.

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