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Role of Reactive Oxygen Species and Nitric Oxide in Gibberellic Acid-Induced Programmed Cell Death of Wheat Aleurone Layer Cells

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ABSTRACT

The important role of nitric oxide (NO) in regulation of reactive oxygen species (ROS) generation and antioxidative isoenzymes activity of wheat aleurone layer was revealed. In the presence of gibberellic acid (GA_3) in the wheat aleurone layer a significant increase of ROS occurred. This effect of GA_3 was associated with maximal decrease of antioxidant enzymes activity. A strong inhibiting effect of NO on GA_3 stimulated the generation of ROS and DNA fragmentation in the wheat aleurone layer. It has been shown that NO prolongs activity of different intracellular forms of wheat aleurone antioxidative enzymes activity by inhibition of oxidative damage to cell. These results suggest that NO has a definite antioxidative role in hormone-regulated programmed cell death of wheat aleurone layer.

Keywords: abscisic acid, ascorbate peroxidase, catalase, DNA laddering, isoenzyme, superoxide dismutase
Abbreviations: ABA, abscisic acid; **APX**, Ascorbate peroxidase; **NO**, nitric oxide; **PCD**, programmed cell death; **ROS**, reactive oxygen species; **SOD**, Superoxide dismutase

INTRODUCTION

During the growth process the aleurone layer of wheat grains synthesize and secrete a number of hydrolytical enzymes (including α-amylase). Induction of synthesis of specific hydrolases depends on the presence of gibberellic acid (GA_3) in tissue. GA_3-dependent hydrolase synthesis is inhibited by the natural antagonist of GA_3 – abscisic acid (ABA) (Bisenbaev *et al.* 1992; Bethke *et al.* 1997) – which results in the elimination of aleurone layer cells during subsequent ontogenesis of this tissue. It was suggested that aleurone cell death is a form of genetically determined cell death or apoptosis (Fath *et al.* 2000; Bissenbaev *et al.* 2004).

It is known that reactive oxygen species (ROS) such as superoxide (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxide radical (OH^-) play crucial roles in the initiation of programmed cell death (PCD) (Gechev *et al.* 2006). Accumulation of these ROS may cause peroxidation of membrane lipids, inactivation of proteins (especially enzymes) and oxidative DNA damage (Sordet *et al.* 2004). The protective mechanisms adapted by plants to scavenge free radicals and peroxides include activation of antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and others. These enzymes are important components in preventing oxidative stress in plants. The process involves SOD catalyzing the dismutation of O_2^- to molecular oxygen and H_2O_2 (Wolfe-Simon *et al.* 2006), and APX and CAT subsequently detoxifying H_2O_2 (Davletova *et al.* 2005). Finally APX reduces H_2O_2 to water, by using ascorbate as an electron donor.

The free radical gas nitric oxide (NO) is a well known signaling molecule in mammalian cells (Moreau *et al.* 2010), and it plays a similar role in plants. It has been implicated in the control of root growth (Lommbardo *et al.* 2006), as an endogenous maturation and senescence factor (Jasid *et al.* 2009), and in the induction of defense responses (Shi *et al.* 2005). As a short-lived, readily diffusible free radical, NO can react with a variety of intracellular and extracellular targets. In some cases, these interactions are cytotoxic and result in cell death. On the other hand, NO acted as an antioxidant and prevented cell death. This protective role of NO may result, in part, from its interaction with lipid hydroperoxyl radicals or highly reactive superoxide, both of which promote lipid peroxidation (Lamattina and Polacco 2007).

The aim of the present investigation was to study the roles of NO and ROS in hormone-regulated programmed cell death of the wheat aleurone layer.

MATERIALS AND METHODS

Plant material: Preparation of wheat aleurone layers

Aleurone layers were isolated from grains of wheat (*Triticum aestivum*, variety Kazakhstanskaya 4) collected from the 2003 harvest. Aleurone layers were prepared from de-embryonated seeds as described previously (Bissenbaev *et al.* 2007) and incubated in media containing 10 mM $CaCl_2$ and 1 μM GA_3 and/or 70-300 μM *S*-nitroso-*N*-acetylpenicillamine (SNAP; Sigma-Aldrich Chemie GmbH, Germany).

DNA extraction and electrophoresis

Aleurone tissues were lysed in a 0.3 ml ice-cold buffer (5 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% (w/v) SDS, 1.4 M NaCl, 0.2 % (w/v) 2-mercaptoethanol) and incubated for 30 min at 60°C. The DNA was extracted with equal volumes of chloroform and after 15 min centrifuged for 10 min at 5000 × *g*. The aqueous phase was transferred to a new tube and 2/3 volumes of ice-cold isopropanol (-20°C) was added and centrifuged for 10 min at 5000 × *g*. The supernatant was discarded, and the pellet containing DNA was dried and resuspended in 500 μl of TE buffer (10 mM Tris-HCl and 0.1 mM EDTA, pH 7.5). An equal volume of phenol: chloroform (1: 1) mixture was added to the final suspension. After centrifugation at 5000 × *g* for 15 min, 1/10 volume sodium acetate

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Original Research Paper

(pH 7.0) was added to the aqueous phase, followed by 2.5 vol of ice-cold ethanol. DNA was allowed to precipitate at –20°C; the pellet containing DNA was dried and resuspended in 50 µl of TE buffer. DNA electrophoresis was carried for 2 h at 70 V in 1.8% agarose gels. DNA was visualized by UV fluorescence after staining with ethidium bromide.

Determination of superoxide radical and hydrogen peroxide

O_2^- was measured as described by Jiang and Zhang (2001), with some modifications, by monitoring nitrite formation from hydroxylamine in the presence of O_2^-. One g of frozen aleurone tissue was homogenized with 3 ml of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5000 × *g* for 10 min. The incubation mixture contained 0.9 ml of 65 mM phosphate buffer (pH 7.8), 0.1 ml of 10 mM hydroxylamine hydrochloride and 1 ml of the supernatant. After incubation at 25°C for 20 min 17 mM sulfanilamide (Sigma-Aldrich) and 7 mM α-naphthylamine (Sigma-Aldrich) were added to the incubation mixture. After reaction at 25°C for 20 min, ethyl ether in the same volume was added and centrifuged at 1500 × *g* for 5 min. The absorbance in the aqueous solution was read at 530 nm. A standard curve with NO_2^- was used to calculate the production rate of O_2^- and hydroxylamine.

The production of H_2O_2 was measured by monitoring the A_{415} of the titanium-peroxide complex following the method described by Brenan and Frenkel (1977). Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2.

Assays of antioxidant enzyme activities

Aleurone layers were ground to a fine powder in liquid N_2, extracted in 300 µl buffer (60 mM K_2HPO_4, pH 7.8, 0.1 mM EDTA, 20 µM E64, 20 µM leupeptin) and the homogenate was centrifuged for 15 min at 4000 rpm at 4°C. Samples of the homogenate were separated on 12.5% native PAGE at 100 V.

SOD (EC 1.15.1.1) activity was assayed using the method as described by Beauchamp *et al.* (1971). After electrophoresis gels were immersed in 2.45 mM nitro blue tetrazolium for 20 min and soaked in a solution containing 28 mM TEMED (tetramethylethylenediamine), 28 µM riboflavin and 36 mM K_2HPO_4, pH 7.8 for 15 min. SOD activity was detected by illuminating the gel which causes it to turn uniformly blue except at positions exhibiting SOD activity. When maximum contrast was achieved, the reaction was stopped by rinsing the gel with H_2O.

APX (EC 1.11.1.11) activity was assayed using the method as described by Mittler and Zilinkas (1993). After electrophoresis the gel was immersed in 50 mM sodium phosphate, pH 7.0 and 2 mM ascorbate for 30 min, changing the solution every 10 min. The gel was soaked in 50 mM sodium phosphate, pH 7.0, 4 mM ascorbate and 2 mM H_2O_2 for 20 min before briefly washing with 50 mM sodium phosphate, pH 7.0. Finally, the gel was incubated in 50 mM sodium phosphate, pH 7.8, 28 mM TEMED and 2.45 mM nitro blue tetrazolium (Sigma-Aldrich) until the gel turned uniformly blue except at positions exhibiting APX activity. When maximum contrast was achieved, rinsing the gel with H_2O stopped the reaction.

Statistical analyses and experimental design

O_2^- and H_2O_2 contents were expressed as µmol per min^{-1} and g^{-1} fresh weight (FW) of aleurone layers. The patterns of responses to GA_3, GA_3 + SNAP or SNAP were reproducible in repeat experiments that were conducted on at least three occasions. Statistical significance of experimental data was determined using the Student's *t*-test. The observed differences between experimental means were statistically significant at $P < 0.05$ and $P < 0.01$.

RESULTS AND DISCUSSION

NO donor inhibit GA_3 induced DNA fragmentation

A characteristic feature of cells undergoing PCD is the formation of DNA laddering as a consequence of the internucleosomal fragmentation of nuclear DNA. For example, the analysis of DNA from GA_3-treated barley and wheat aleurone cells detected DNA laddering (Whang *et al.* 1996; Bissenbaev *et al.* 2004; Domínguez *et al.* 2004). In this study we investigated the effect of SNAP, a donor of NO, on the GA_3-stimulated fragmentation of DNA in wheat aleurone layer. **Fig. 1** shows that GA_3, at 1 µM, stimulated DNA laddering of wheat aleurone cells. The data supports our previous proposition that the apoptotic character associated with wheat aleurone cell death is initiated by GA_3 (Bissenbaev *et al.* 2004). This process is accompanied by internucleosomal DNA fragmentation, a recognised hallmark of apoptosis (Nagata 2005). The addition of 75 µM SNAP to the incubation medium did not influence the GA_3-stimulated fragmentation of wheat aleurone layer cells. Increasing the concentration of SNAP up to 150 and 300 µM significantly decreased GA_3-induced fragmentation of DNA to oligonucleosomal fragments (**Fig. 1**, lanes 3-4).

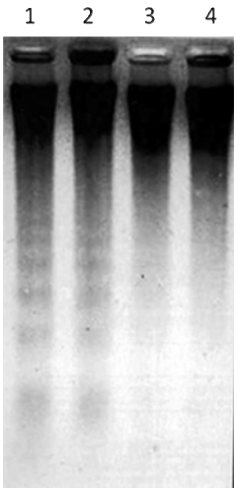

Fig. 1 Effect of GA_3 and SNAP on the fragmentation of DNA of wheat aleurone layer cells incubated for 72 hrs. 1 – GA_3 (1 µM); 2 – GA_3 (1 µM) + SNAP (75 µM); 3 – GA_3 (1 µM) + SNAP (150 µM); 4 – GA_3 (1 µM) + SNAP (300 µM).

The experiments reported here on the effect of NO on DNA fragmentation in wheat aleurone cells have parallels with a similar study on the effect of NO on GA_3-induced PCD in barley aleurone cells (Beligni *et al.* 2002). It has been shown that the NO donor sodium nitroprusside (SNP) delays PCD in barley aleurone layers, which is determined by vital staining with fluorescein diacetate (FDA) and *N*-(3-triethylammoniumpropyl)-4-(6-[4-(diethylamino) phenyl] hexatrienyl) pyridinium dibromide or by monitoring net O_2 consumption. In addition to the data mentioned above our results suggest that NO delays GA_3-induced PCD in wheat aleurone cells as detected by DNA fragmentation.

Effect of NO on ROS accumulation

Previously we showed that the generation of ROS is strongly correlated with GA_3-induced DNA fragmentation of wheat aleurone cells (Bissenbaev *et al.* 2007). These data suggests that ROS generation is a necessary link to initiate a response cascade that results in PCD. Various abiotic stresses (drought, extreme temperatures, UV, etc.) may induce ROS generation. ROS initiates the oxidation processes, leading to cell destruction and may also serve to trigger an array of signal pathways. Thus the maintenance of appropriate levels of intracellular ROS may in some measure represent an ability to aid survival. NO interacts differently with ROS and is able to exhibit antioxidant function under stress (Besson-Bard *et al.* 2008; Gadjev *et al.* 2008).

Since NO is a toxic-free radical, most of the work on NO function in plant cells has focused on its ability to act in the same direction as active oxygen species (Hong *et al.* 2008). In animal systems, several reports convincingly

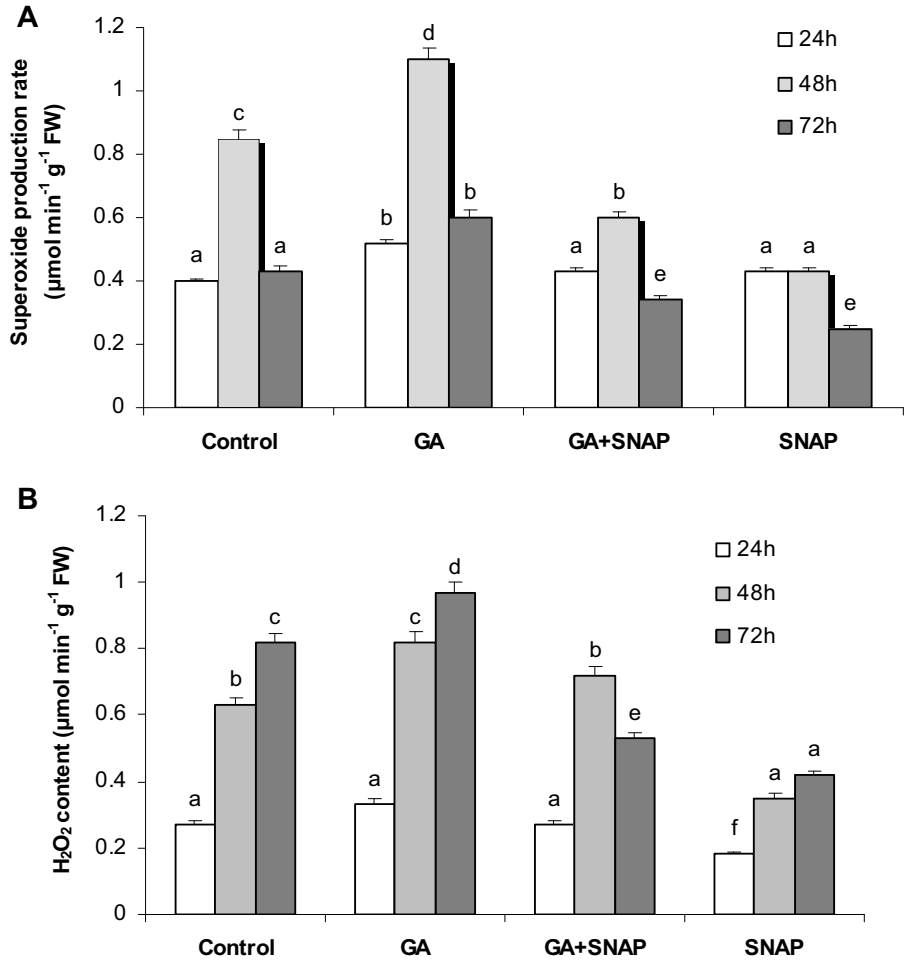

Fig. 2 Effect of GA_3 and SNAP on the generation of ROS in wheat aleurone layer cells. Effect of GA_3 and SNAP on the generation of O_2^- in wheat aleurone layer cells (**A**). 1-24 hrs; 2 – 48 hrs; 3 - 72 hrs; Dosage of GA_3: 1 µM; Dosage of SNAP: 300 µM. Effect of GA_3 and SNAP on the generation of H_2O_2 in wheat aleurone layer (**B**). 1-24 hrs; 2 – 48 hrs; 3 – 72 hrs; Dosage of GA_3: 1 µM; Dosage of SNAP: 300 µM. Values are the means of three different experiments. Error bars represent SE with n = 4.

demonstrate a protective role of NO in oxidative stress (Borniquel *et al*. 2006; Patwari and Lee 2007; Zhang *et al*. 2007). In a series of work conducted by Lamattina's group, it was found that NO protects against cellular damage produced by diquat, paraquat and *Phytophthora infestans* in potato leaves, including PCD in the barley aleurone layer (Lamattina and Polacco 2007). However, until now, the direct effects of NO on the accumulation of ROS in cereal aleurone layer were not known.

Fig. 2 shows the effect of SNAP on ROS content of wheat aleurone layers treated with GA_3. It was revealed that the presence of 1 µM GA_3 in the incubation medium for 48 hrs significantly increased O_2^- generation in comparison to controls (**Fig. 2A**). Further elongation of incubation time with GA_3 (72 hrs) led to a decrease of O_2^- concentration in aleurone layer cells in comparison to 48 hrs of incubation. However, under these conditions the O_2^- concentration was approximately 30% higher compared to the control. These GA_3-induced effects were significantly blocked when 300 µM SNAP was added to the incubation medium.

The study of hormone-dependent accumulation of H_2O_2 in aleurone layer cells showed that in the absence of GA_3 and SNAP the intracellular concentration of H_2O_2 increased as the incubation time was extended. Significant increases of H_2O_2 concentration occurred after 48 hrs of incubation. The incubation of aleurone layer tissue in 1 µM GA_3 for 24 and 48 hrs caused an increase in intracellular concentration of H_2O_2 to 0.33 and 0.82 µM, respectively. The presence of GA_3 in incubation medium for 72 hrs led to a considerable increase of H_2O_2 concentration in wheat aleurone layers, and reached 0.97 µM. The inclusion of SNAP to incubated aleurone layers (300 µM) drastically abolished the effect of GA_3 for all incubation intervals and this was a similar observation with the O_2^-. These results suggest a key role of NO in the regulation of hormone-dependent generation of oxygen radicals. The substantial inhibiting effect of NO both on H_2O_2 and O_2^- generation may indicate its antioxidant function in this model system.

NO donor delays the GA_3-stimulated loss of antioxidant enzymes

The protective effect of donor NO on GA_3-stimulated PCD of wheat aleurone layer tissue is clearly associated with the decrease in ROS (**Fig. 2**). Plant cells are equipped with several free radical detoxifying enzymes to protect them against oxidative damage (Davletova *et al.* 2005; Myouga *et al.* 2008). These enzymes include SOD, APX, CAT and others.

GA_3-induced PCD in wheat aleurone layers is preceded by a decrease in the activity of enzymes that metabolize ROS and this leads to an increase in the content of intracellular ROS. In contrast, ABA stimulates the activity of antioxidant enzymes and, as a consequence, suppresses production of ROS in the wheat aleurone layer (Bissenbaev *et al.* 2007). This might suggest an inhibiting effect of NO on GA_3-stimulated ROS generation, as in the case with ABA, and may be linked with increased activity of antioxidant enzymes in wheat aleurone tissue.

As shown in **Fig. 3** the activity of SOD in the aleurone layer decreased in the presence of GA_3 as incubation time increased. The substantial inhibiting effect of GA_3 on SOD activity occurred prior to the completion of the 48th hour of incubation. After 72 hrs of incubation with GA_3 the activity

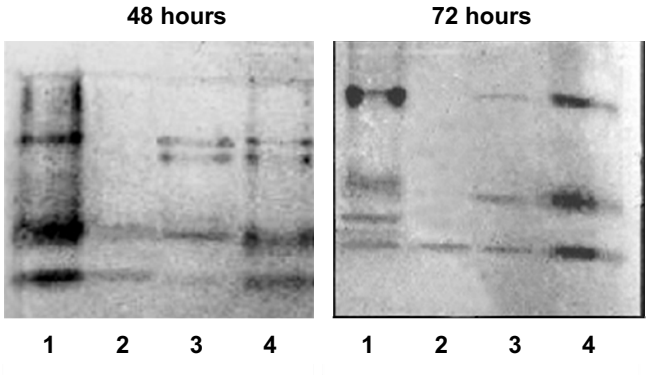

Fig. 3 Effect of GA_3 and SNAP on the isoenzymes of superoxide dismutase of wheat aleurone layer cells. Aleurone cells were incubated in GA_3 and/or SNAP for the indicated time and homogenized. 40 μg of proteins were loaded for each lane. Clear areas of the gel indicate the presence of enzyme activity. 1 – control; 2 – GA_3 (1 μM); 3 – GA_3 (1 μM) + SNAP (300 μM); 4 – SNAP (300 μM).

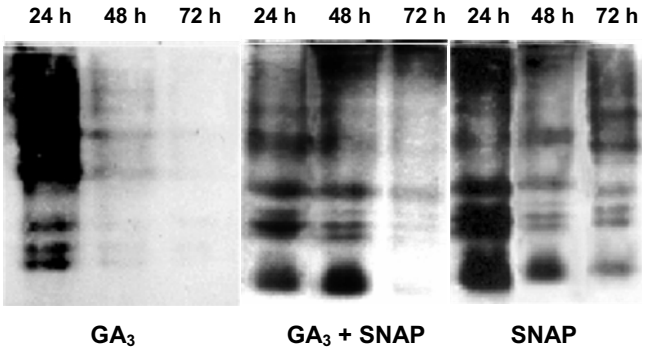

Fig. 4 Effect of GA_3 and SNAP on the isoenzymes of ascorbate peroxidase of wheat aleurone layer cells. Aleurone cells were incubated in GA_3 and/or SNAP up to 72 hrs and homogenized. 40 μg of proteins were loaded for each lane. Clear areas of the gel indicate the presence of enzyme activity. Dosage of GA_3: 1 μM; Dosage of SNAP: 300 μM.

of SOD was represented on an electrophoregram by only one electronegative protein zone with Cu/Zn-SOD activity. Conversely, the incubation of aleurone layers in the presence of GA_3 (1 μM) and SNAP (300 μM) for 48 hrs resulted in high activity level of SOD in comparison to cells incubated in the presence only of GA_3 (1 μM) alone. At the same time after 72 hrs of incubation, SNAP did not exert a noticeable effect to GA_3-mediated suppression of SOD isoenzymes. The addition of 300 μM SNAP alone to incubated aleurone cells preserved SOD activity up to 72 hrs.

In further experiments we then studied the effect of GA_3 and SNAP on the activity of APX in the wheat aleurone layer (**Fig. 4**). The incubation of isolated aleurone layer cells with GA_3 (1 μM) for 48 hrs substantially inhibited the activity of APX isoenzymes. After 72 hrs of incubation in the presence of GA_3, the activity of APX was not detected on an electrophoregram.

Analysis of the temporal effect of NO on GA_3 action showed that NO arrested the inhibiting effect of this phytohormone. In similar observations with SOD even 72 hrs of incubation with SNAP produced no significant effect on GA_3-induced inhibition of APX (**Fig. 3**). These data suggest the important role of NO in regulation of antioxidant enzyme activities in wheat aleurone layer cells. However, NO, in contrast to ABA, does not completely block the ability of GA_3 to inhibit activities of SOD and APX, but only temporarily detains the effect of the said phytohormone.

CONCLUDING REMARKS

PCD in intact cereal aleurone layer tissue is prevented by ABA and stimulated by GA_3 (Fath *et al.* 2000; Bissenbaev *et al.* 2004). ABA can also delay death of aleurone cells that have been treated with GA_3. In this report, we extend our observations on PCD to show that NO can selectively delay the death of GA_3-treated wheat aleurone cells.

It is clear that in our model system NO exhibits an antioxidant function. This is corroborated by the drastic inhibition of GA-induced generation of oxygen radicals in aleurone tissue in the presence of NO, independent of incubation time with the phytohormone. However, the inhibiting effect of NO on oxygen radical generation might not be connected with the activation of antioxidant enzymes. It is likely that in aleurone layer cells NO do not directly activate SOD and APX, but only enhances the functional endurance of these enzymes, and thereby prevents oxidative damage to the cells.

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