Role of reactive oxygen species and antioxidant enzymes in hormone regulating programmed cell death of wheat aleurone layer

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Abstract

Hormonal regulation of reactive oxygen species and antioxidant enzyme production in wheat aleurone layer was revealed. In the presence of gibberellic acid in wheat aleurone layer significant increase of reactive oxygen species was observed. This effect of gibberellic acid was associated with maximal decrease in the antioxidant enzyme activity. The present study showed that in the given model system influence of abscisic acid was directed to the suppression of ROS production through increase in antioxidant enzyme synthesis. Strict correlation between progression of wheat aleurone programmed cell death, ROS production and changes in activity of antioxidant enzymes was revealed. Relying on the obtained data it was suggested that ROS and antioxidant enzymes could play important roles in realization of hormone induced programmed cell death of wheat aleurone layer.

Key Words: Aleurone layer, plant hormone, superoxide, superoxide dismutase, ascorbate peroxidase.
quality of cereals, and other) (Young et al., 1997).

During their growing process aleurone layer of wheat grain synthesize and secrete number of hydrolytical enzymes (including α-amylase). Induction of synthesis of the given hydrolases depends on the presence in tissue gibberellic acid (GA). GA-depending hydrolases synthesis is inhibited by natural antagonist of the GA – abscisic acid (ABA) (Bissenbaev et al., 1992; Bethke et al., 1997). On the subsequent ontogenesis stages wheat aleurone layer cells are eliminated. It is suggested that aleurone cell death is programmed, or genetically determined (apoptosis) (Fath et al., 2000; Bissenbaev et al., 2001; Bissenbaev et al., 2004).

It is known that reactive oxygen species such as superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxide radical (OH) play crucial role in initiation of programmed cell death (PCD) (Jab, 1999).

Accumulation of these reactive oxygen species may cause peroxidation of membrane lipids, inactivation of proteins (enzymes) and oxidative DNA damage (Sordet et al., 2004). The protective mechanisms adapted by plants to scavenge free radicals and peroxides include antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidases (APX), catalases (CAT) and others. These enzymes are important components in preventing the oxidative stress in plants. SOD catalyzes the dismutation of superoxide radical to molecular oxygen and H$_2$O$_2$ (Bawler et al., 1994). APX and CAT then detoxify the produced H$_2$O$_2$ (Mittler et al., 1998). APX reduces H$_2$O$_2$ to water, by using ascorbate as an electron donor.

The aim of the present investigation was to investigate the roles of reactive oxygen species and antioxidant enzymes in hormone induced programmed cell death of wheat aleurone layer.

**Materials and methods**

**Plant material: Preparation of wheat aleurone layers**

Wheat (*Triticum aestivum*, variety Kazakhstanskaya 4) seed aleurone layers isolated from grains of 2003 harvested. Aleurone layers were prepared from deembryonated seeds as described previously (Bissenbaev et al., 2004) and incubated in media containing 10mM CaCl$_2$ and 1μM GA and/or 5μM ABA.

**DNA extraction and electrophoresis**

Aleurone tissues were lysed in a 0,3 ml ice cold buffer (10 mM Tris-HCl (pH 7.2), 10 mM EDTA, 5 mM 2–mercaptoethanol, 0.5% Triton X-100) (5 mM Tris-HCl (pH 8.0), 20 mM EDTA, 2 % (w/v) sodium dodecylsulphate (SDS), 1.4 M NaCl, 0.2 % (w/v) 2–mercaptoethanol) and incubated for 30 min at 60°C.

Extraction of DNA was carried out according to Young and Gallie (1999) with some modifications. The DNA was extracted with equal volumes of chloroform and after 15 min centrifuged for 10 minutes 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.

Determination of superoxide radical and hydrogen peroxide

O$_2^-$ was measured as described by Jiang and Zhang (Jiang et al., 2001) by monitoring the nitrite formation from hydroxylamine in the presence of O$_2^-$, with some modifications. One gram of frozen aleurone tissue leaf segments was homogenized with 3 ml of 65 mM potassium phosphate buffer (pH 7.8) and centrifuged at 5,000 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 and 7 mM α-naphthylamine were added to the incubation mixture. After reaction at 25°C for 20min, same volume of ethyl ether in the 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 content of H₂O₂ was measured by monitoring the A₄₁₅ of the titanium-peroxide complex following the method described by Brenan and Frenkel (Brenan et al., 1977). Absorbance values were calibrated to a standard curve generated with known concentrations of H₂O₂.

Assays of antioxidant enzyme activities

Extraction of antioxidant enzymes was carried out according to Jiang and Zhang (2001) with some modifications. Aleurone layers were ground to a fine powder in liquid N₂, extracted in 300 µl buffer (60 mM K₂HPO₄, 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 activity was assayed using the method as described (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 K₂HPO₄ (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, rinsing the gel with H₂O stopped the reaction.

APX activity was assayed using the method as described (Mittler et al., 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 H₂O₂ 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 until the gel turned uniformly blue except at positions exhibiting APX activity. When maximum contrast was achieved, rinsing the gel with H₂O stopped the reaction.

Results and discussion

Study of the time course of GA’s action on the DNA fragmentation of aleurone layer cells showed that treatment of 1 µM GA significantly enhanced DNA fragmentation only after incubation of aleurone layer for 72 hour. Subsequent increase of incubation time

![Figure 1](image-url)
was accompanied with significant enhancement of DNA fragmentation. Under these conditions different sized fragments formed characteristic internucleosomal ladders when on agarose gel electrophoresis (Figure 1A). Addition of 0.001-1µM ABA to the incubation medium entirely abolished GA-stimulated fragmentation of DNA to oligonucleosomal fragments.

These data support our previous suggestions about apoptotic characters of wheat aleurone cell death that are initiated by GA. It seems probably that wheat aleurone cell death is initiated in 48 hours after GA treatment and it reaches maximal level in 96 hours after treatment. This process accompanied by internucleosomal DNA fragmentation that is a critical hallmark of apoptosis (Gilchrist, 1998).

ROS are the products of cellular metabolism. In plant cells the production of ROS has been shown in mitochondria, chloroplasts, the plasma membrane, peroxisomes and the apoplastic space. ROS may also be generated by ionizing or ultraviolet radiation. Equally, certain exogenous chemicals may redox cycle following metabolism by the cell, with the subsequent production of electrons that can be transferred to molecular oxygen producing superoxide. Irrespective of their origin, reactive oxygen species may interact with cellular biomolecules, such as protein, lipids and DNA, leading to modification and potentate cell death. However, ROS can be produced specifically as part of programmed cell death (Jab, 1999).

The initial experimental evidence that ROS also acted as a signal in plant PCD was obtained in cell suspensions by demonstrating that \( \text{H}_2\text{O}_2 \)-induced cell death could be blocked by cycloheximide and protease inhibitors (Levine et al., 1994). It has been shown that two mutants of Arabidopsis, lesion-stimulating disease1 (lsd1) and radical-induced cell death1 (rcd1), elevate ROS levels are necessary and sufficient to induce spreading of cell death (Jabs et al., 1996; Overmyer et al., 2000; Mateo et al., 2004). It was demonstrated that the conditional fluorescent (flu) mutant of Arabidopsis that generates singlet oxygen upona dark-to-light shift initiate a cell death response immediately after the release of singlet oxygen (Camp et al., 2003).

These data suggests that ROS generation is a necessary link to initiate a response cascade that results in cell death. Among the questions that remain...
to be resolved about ROS in plants, especially in the context of cell death, are identities of the ROS species that participate in PCD.

Therefore in our following experiments the time course of GA’s action on the accumulation of superoxide and peroxide radicals in the wheat aleurone layers were investigated. It was revealed that presence of 1 µM GA in incubation medium for 24 and 48 hours significantly increased superoxide production, when compared with the control (Figure 2).

At 72 h, the levels of superoxide decreased significantly, and returned almost to the level of control. Addition of 5 µM ABA to the incubation medium entirely abolished GA-stimulated accumulation of superoxide in the wheat aleurone layer within the all time of GA treatment. The presence of 5 µM ABA alone significantly decreased the level superoxide compared with control and with the effect of GA within the all time of incubation.

The study of hormone dependent accumulation of H$_2$O$_2$ in aleurone layer showed that presence 1 µM GA in incubation medium resulted in continuous increase in the level of H$_2$O$_2$ within all time of incubation (Figure 3). Addition of 5µM ABA to the incubation medium entirely blocked GA-stimulated accumulation of H$_2$O$_2$ in the wheat aleurone layer within the all time of GA treatment.

Thus, these data indicate that in the presence of GA in aleurone layer the processes of superoxide and peroxide production were really increased. Also most significant superoxide anion accumulation was observed in 48 hours after GA treatment. Whereas GA-depended H$_2$O$_2$ content in the aleurone layer reached its maximal point in 72 hours of incubation.

It is known that ROS are products of cell metabolism and their intracellular level is regulated by number of antioxidant enzymes (superoxide dismutase, aspartate peroxidase, catalase and others).

As mentioned above, SOD catalyzes the dismutation of superoxide radical to molecular oxygen and more less toxic H$_2$O$_2$. More than 80% of SOD activity is identified in cytosol, and other 20% - in organelles, mainly in mitochondria (Bawler et al., 1994). APX and CAT then detoxify the produced H$_2$O$_2$.

This might suggest that observed differences in production of superoxide and H$_2$O$_2$ to GA response is
supplied by dismutation of superoxide-anion into 
\( \text{H}_2\text{O}_2 \) through activation of superoxide dismutase 
reaction. A noticeable fact that is increases of cellular 
concentration of \( \text{H}_2\text{O}_2 \), which was produced as a result 
of superoxide dismutase reaction, can be dangerous to the cell itself because of hydroxide radical production probability. That is why \( \text{H}_2\text{O}_2 \) must be constantly inactivated in the reaction, which is catalyzed by ascorbate peroxidase and/or catalase (Shigeoka et al., 2002).

Therefore in subsequent experiments we have investigated the effects of GA and ABA on the activation pattern of wheat aleurone layer antioxidant enzymes, especially SOD and APX.

As it is seen in Figure 4, in the presence of GA activity of SOD isoenzymes of aleurone layer decreased in correlation with incubation time. Insignificant suppressing effect of GA was observed in 48 hour after incubation. After 72 hours of incubation, electrophoretic SOD activity almost disappeared and was presented only by one electronegative protein zone with SOD activity. Analysis of time-dependent ABA effect demonstrated that ABA strengthens the activity of all intracellular SOD forms independently from duration of incubation time. ABA introduction in 5 \( \mu\text{M} \) dosage into aleurone layers incubating with GA significantly blocked inhibitory effect of GA on activity of intracellular SOD forms.

As it can be seen in Figure 5, in the GA presence activity of aleurone layer APX isoenzymes were decreased in correlation with increase of incubation time. Incubation of isolated aleurone layer with GA(1 \( \mu\text{M} \)) for 48 hours significantly inhibited the activity of APX isoenzymes. After 72 hours of incubation with GA activity of APX isoenzymes was not observed on electropherogram. Analysis of time-dependent ABA effect demonstrated that ABA strengthened the activity of all intracellular types of APX. ABA introduction in 5 \( \mu\text{M} \) dosage into culture of aleurone layers, incubating with GA, significantly blocked the inhibitory effect of GA on activity of intracellular APX forms of wheat seed aleurone layers.

Obtained data indicate the important role of hormones in regulation of antioxidant enzymes of wheat seed aleurone layer. ABA stimulates the activity of antioxidant enzymes and, as a consequence, suppresses production of ROS in wheat aleurone layer. In contrast, influence of GA in the given model system

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**Figure 4.** Effect of GA and ABA on the isoenzymes of superoxide dismutase of wheat aleurone layer (Dosage of GA: 1 \( \mu\text{M} \), Dosage of ABA: 5 \( \mu\text{M} \); Mn: mitochondrial SOD, Fe: Plastid SOD; CZ: cytosolic SOD)
directed to suppress the activity of antioxidant enzymes. Also the maximal effect of GA on APX and SOD activity was observed in three days after incubation, which indicates the direct correlation between decrease antioxidant enzymes activity, enhancement of ROS production and progression of aleurone cells PCD, which identified by oligonucleosomal degradation of DNA.

**Conclusions**

Presented results may indicate the key role of ROS and antioxidant enzymes in hormone induced PCD of wheat aleurone layer. GA strengthens apoptosis, which is followed by oligonucleosomal DNA fragmentation – the critical apoptosis hallmark. Probably, before PCD intracellular concentration of ROS is increased due to GA-dependent inhibition of enzymes of ROS metabolism. Decrease of ROS metabolizing enzyme’s activity in GA treated cells significantly strengthens the process of accumulation of ROS and PCD of aleurone layer. In contrast, during the incubation of aleurone layers in the presence of ABA they do not undergo PCD (Bissenbaev et al., 2001) and they have high level of ROS metabolizing enzymes activity in comparison with cells, which were incubated only in the presence of GA.

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**References**


