J. Plant Biol. (2010) 53:205–213
DOI 10.1007/s12374-010-9106-y

ORIGINAL RESEARCH

Inhibition of Oxidative Phosphorylation Induces a Rapid Death of GA-Pretreated Aleurone Cells, But Not of ABA-Pretreated Aleurone Cells

Hyeokgon Park · Minji Park · Hui-kyeong Yim · Seunghye Park · EonSeon Jin · Yong-sic Hwang

Received: 14 February 2010 / Revised: 8 April 2010 / Accepted: 11 April 2010 / Published online: 25 May 2010

Abstract Reactive oxygen species (ROS) mediate programmed cell death in aleurone cells, which is promoted by gibberellic acid (GA) and prevented by abscisic acid (ABA). Plant mitochondria contain two distinct respiratory pathways: respiration through cytochrome c oxidase increases ROS production, whereas respiration through the alternative oxidase pathway lowers it. While studying the effects of GA and ABA on partitioning of respiration between those two pathways during the germinating process, we discovered that oxidative phosphorylation inhibitors like sodium azide and 2, 4-dinitrophenol induce rapid death of GA-pretreated aleurone cells but not of ABA-pretreated cells. Functional aerobic respiration was required for GA signaling, and 6 to 12 hours of GA signaling altered the cellular state of aleurone cells to be extremely susceptible to inhibition of oxidative phosphorylation. Anaerobic conditions were also able to mimic the effects of respiratory inhibitors in specifically inducing cell death in GA-treated cells, but cell death was provoked much more slowly. Cotreatment with various antioxidants did not prevent this process at all, suggesting that no ROS are responsible for this respiratory inhibitor-induced cell death. Our observation implicates that GA may partition all the electrons produced during mitochondrial respiration only to the cytochrome oxidase pathway, which would at least partly contribute to cellular accumulation of ROS.

H. Park · M. Park · H.-k. Yim · Y.-s. Hwang (✉)
Department of Bioscience and Biotechnology, Konkuk University,
1 Hwayang-dong, Gwangjin-gu,
Seoul 143-701, Republic of Korea
e-mail: yshwang@konkuk.ac.kr

S. Park · E. Jin
Department of Life Science, College of Natural Science,
Hanyang University,
Seoul 133-791, Republic of Korea

Keywords Aleurone · Cytochrome c oxidase · Alternative oxidase · Oxidative phosphorylation · Cell death · Respiration rate

Abbreviations

GA	gibberellic acid
ABA	abscisic acid
DNP	2, 4-dinitirophenol
FDA	Fluorescein di-acetate
FM4-64	*N*-(3-triethylammoniumpropyl)-4-{6-[4-(diethylamino) phenyl]-hexatrienyl} pyridinium dibromide
SHAM	Salicylhydroxamic acid

Introduction

Before their phototrophic capabilities are established, plant seedlings must rely on the nutrient reserve stored in the seed to support the growth of the embryonic axis. In cereal plants, aleurone cells help mobilize the starchy endosperm reserves by synthesizing and secreting a variety of hydrolytic enzymes (Ritchie et al. 2000). After accomplishing its role as a specialized secretory tissue, aleurone cells are developmentally programmed to die (Fath et al. 2000). Gibberellic acid (GA) induces the synthesis and secretion of various hydrolytic enzymes in aleurone cells, thus promoting cereal germination, whereas abscisic acid (ABA) inhibits the action of GA at various steps, which slows down the germination process. In response to GA, aleurone cells transcribe a large spectrum of hydrolase genes (Lovegrove & Hooley 2000) and also increase the amino acid pool of the cell by mobilizing storage proteins in protein storage vacuoles (PSVs), which provides the building blocks for de novo synthesis of various hydrolytic

enzymes (Bethke et al. 1998; Hwang et al. 2003). Along with these molecular responses, aleurone cells undergo prominent cellular changes. For example, unstimulated aleurone cells are highly packed, with a large number of the smaller PSVs (Bethke et al. 1998). However, once stimulated by GA, the cells undergo vacuolar acidification (Swanson & Jones 1996) and vacuolar fusion, establishing a large central vacuole. Highly vacuolated cells eventually lose plasma membrane integrity, resulting in cell death. GA is known to promote this programmed cell death (PCD) process in aleurone cells, while ABA prevents it (Bethke et al. 1999).

Not much, however, is known about how GA induces PCD in barley aleurone cells. GA-induced PCD of barley aleurone cells is distinguished from apoptosis because the cells do not undergo characteristic apoptotic changes in morphology and chromosomal DNA organization, suggesting that it occurs by a distinct mechanism (Fath et al. 1999). Previously, GA-treated aleurone cells were found to be more susceptible to exogenous or endogenous H_2O_2 than ABA-treated cells, implicating reactive oxygen species (ROS) in the cell death pathway of barley aleurone cells (Bethke & Jones 2001). GA has been demonstrated to increase the sensitivity of aleurone cells to ROS by reducing ROS scavenging activity. For example, GA decreases the steady-state messenger RNA (mRNA) and protein levels of enzymes such as catalase, ascorbate peroxidase, and superoxide dismutase, and so on, whereas ABA maintains them (Fath et al. 2002; Fath et al. 2001). Therefore, it was hypothesized that the ROS produced in GA-treated aleurone cells may cause cell death by disrupting the plasma membrane (Bethke et al. 2002).

In addition to decrease in ROS scavenging activity, increased ROS production also would contribute to the accumulation of ROS. A few sites of ROS generation can be considered potentially involved in GA-induced PCD of aleurone cells. ROS generation from plastids seems unlikely because aleurone cells, which are nonphotosynthetic, do not have well-developed plastids (Jones 1969). Glyoxysomes, which are a site of active lipid metabolism, may be a potential source because fatty acid oxidation can produce large amounts of ROS as a byproduct (Corpas et al. 2001; Mori et al. 2009). In animals and plants, mitochondria are believed to be a major site of ROS production because the production of ROS is an unavoidable consequence of aerobic respiration (Fleury et al. 2002). For example, in the conventional mitochondrial electron transport chain, cytochrome c oxidase reacts with oxygen molecules, which generates H_2O. However, an oxygen molecule can occasionally react with other electron transport components to form the superoxide anion (O_2^-). A single electron transfer to molecular oxygen converts it to a superoxide anion, and it has been estimated that 1 to 2% of oxygen consumption in the cell produces superoxides (Puntarulo et al. 1988). Through a variety of reactions, superoxides lead to the formation of hydrogen peroxide, hydroxyl radicals, and other ROS, any of which can damage cells in various ways (Møller 2001).

Plant cell respiration, in addition to a conventional cytochrome oxidase pathway, has the unique feature of having an alternative respiratory pathway, mediated by an alternative oxidase (AOX). In contrast to the cytochrome oxidase pathway, which allows efficient ATP synthesis by coupling the reduction of O_2 to proton motive force, the AOX pathway does not provide cells with any energetic benefit. AOX can react with oxygen molecules to form H_2O, but most of the free energy released during electron flow through this enzyme is lost as heat and cannot be used for ATP synthesis (Juszczuk & Rychter 2003). Thermogenesis during flowering in Araceae is known to occur through electron transport via AOX (Vanlerberghe & McIntosh 1997). However, the ubiquity of AOX in most plants that are not generally thermogenic suggests a more general role for the enzyme in limiting mitochondrial ROS formation (Popov et al. 1997; Purvis 1997; Wagner & Moore 1997). The AOX pathway bypasses those electron transfer steps that can generate the superoxide and thereby functions to regulate electron overflow by sharing the ubiquinone pool with the cytochrome oxidase pathway (Maxwell et al. 1999).

AOX respiration is known to be predominant in the early stages of germination (Yentur & Leopold 1976). Because the efficient cytochrome-mediated respiratory chain has not yet been established in newly imbibed seeds, oxidation of cytosolic NADH through AOX would allow recycling of the NAD^+ required to maintain glycolysis. In this way, the plant can avoid shifting to fermentative metabolism, which can have deleterious consequences (Botha et al. 1992). The respiratory transition from the AOX pathway to the cytochrome oxidase pathway has been reported to occur in the seeds of many monocots and dicots as the germinating process proceeds (Esashi et al. 1981; Yentur & Leopold 1976). Because the cytochrome oxidase and AOX pathways have opposite influences on ROS production and because the balance between the two pathways may change during germination, we sought to investigate whether the respiratory transition in aleurone cells is controlled by the phytohormones GA and ABA, which have mutually antagonistic effects on germination. During our study, we discovered that GA somehow alters the cellular state of aleurone cells to be extremely sensitive to oxidative phosphorylation inhibitors, whereas ABA makes the cells more resistant. In the present study, the specific effects of oxidative phosphorylation inhibitors and anoxia on aleurone cell viability and GA response are examined and characterized.

Materials and Methods

Aleurone Layer Preparation

Embryoless half seeds were prepared by cutting off the embryo part of barley seeds (*Hordeum vulgare* cv. Himalaya). Twenty to one hundred embryoless half seeds were surface-sterilized by washing in 7% bleach for 20 minutes with shaking at 125 rpm, and the bleach was completely removed by washing with sterile ddH_2O more than four times. Hypochlorite was neutralized by incubating the half seeds in 0.01 N HCl for 10 minutes. Finally, the seeds were washed again with ddH_2O more than four times and spread on Whatman filter paper containing 5 to 7 ml of filter-sterilized l-arginine solution (50 mM). After 2-day incubation at 25°C, the aleurone layers were isolated by squeezing out the starchy endosperm.

Chemical and Anoxia Treatment of Aleurone Layers

For phytohormone treatments, isolated aleurone layers were treated with 10 μM GA or 20 μM ABA (a mixture of *cis*- and *trans*-ABA) in 20 mM $CaCl_2$ solution. For the no hormone control, aleurone layers were incubated in 20 mM $CaCl_2$ solution. About 20 to 40 layers were placed in a 25 ml Erlenmeyer flask containing 1.5 to 2.5 ml of incubation solution for the indicated times at room temperature with orbital shaking at 50 rpm.

GA- or ABA-pretreated and no hormone-treated layers were further treated with oxidative phosphorylation inhibitors (5 mM sodium azide, 2.5 mM 2, 4-dinitrophenol [DNP]) or AOX inhibitor (2 mM salicylhydroxamic acid) in 20 mM $CaCl_2$ solution for 30 minutes. For antioxidant experiments, GA- or ABA-pretreated aleurone layers were cotreated with oxidative phosphorylation inhibitor and various antioxidants such as butylated hydroxytoluene (BHT), dithiothreitol (DTT), and ascorbate.

For anoxic treatments, aleurone layers of each sample were submerged in 20 mM $CaCl_2$ solution under flushing with N_2 gas and incubated for the indicated times.

Determination of Cell Viability Through Fluorescence Microscopy

After each treatment, aleurone layers were stained with 1 μM fluorescein di-acetate (FDA, Sigma) and 20 μM of *N*-(3-triethylammoniumpropyl)-4-{6-[4-(diethylamino) phenyl]-hexatrienyl} pyridinium dibromide (FM4-64) (Molecular Probes) in 20 mM $CaCl_2$ for 10 minutes, and excessive staining solution was removed by washing the layers in 20 mM $CaCl_2$.

The fluorescence of the sample was observed using a Zeiss Axioskop fluorescence microscope (Zeiss, Shinjuku, Tokyo, Japan), with an AttoArc 2 fluorescence lamp. Excitation BP 450–490 mode was employed for experiments. Images were captured using a Hamamatsu C8800-01C digital camera (Hamamatsu, Shizuoka, Japan).

Viable cells were counted based on their green fluorescence and expressed as a percentage of the total number of cells captured in an image. At least three images captured from independent experiments were employed for counting.

α-Amylase Enzyme Assay

Five aleurone layers were incubated in 2 ml $CaCl_2$ solutions of each treatment, and 100 μl of the media was used for assaying α-amylase activity. Alpha-amylase activity was determined by the starch-iodine method (Jones 1967).

Results and Discussion

Sodium Azide, A Cytochrome C Oxidase Inhibitor, Can Induce Rapid Death of Cells Preincubated With GA, But Not of ABA-pretreated Cells

The aleurone layer is an ideal system to examine the respiratory transition between the AOX pathway and the cytochrome oxidase pathway because of the simple and homogenous nature of the tissue and efficient access of exogenous chemicals to the cells. To examine the effects of GA and ABA on respiratory activity mediated by the cytochrome c oxidase, aleurone cells were preincubated with either hormone and then treated with a cytochrome c oxidase inhibitor, sodium azide (NaN_3). Surprisingly, we noticed that NaN_3 induced rapid curling of the aleurone layers pretreated with GA for 23 hours, indicating rapid loss of cell turgor. In measurements of the respiration rate, GA-pretreated aleurone cells show complete inhibition of oxygen uptake within a few minutes of NaN_3 addition, whereas ABA-treated cells were still able to consume oxygen under the same conditions (data not shown).

Fluorescein diacetate (FDA) is an acetylated form of the green fluorescent dye fluorescein. Acetylation of the fluorescein renders the dye cell-permeable and nonfluorescent. Once FDA enters the cytoplasm, it can be deacetylated by nonspecific esterases and retained inside the cell due to its charge groups. The resultant fluorescence of the cytoplasm from high esterase activity is a convenient indicator for viable cells and has been conventionally used as an assay of cell viability (Boyd et al. 2008; Jones & Senft 1985). Each hormone-treated aleurone cell sample was further treated with NaN_3 for 30 minutes and then stained with FDA for 10 minutes to check viability. As

indicated in Fig. 1a, aleurone cells preincubated with GA for 23 hours show establishment of a large central vacuole and rapidly lose their fluorescence upon addition of NaN_3 (Fig. 1b). However, ABA-preincubated cells do not form large vacuoles and still display high fluorescence under the same conditions, indicating that most of them are viable and still metabolically active.

The cytoplasm of newly imbibed aleurone cells is almost filled with numerous PSVs, often referred to as aleurone grains (Bethke et al. 1998). PSVs contain minerals and storage proteins that become hydrolyzed by vacuolar proteases to provide the amino acids necessary to produce secretory hydrolases (Bethke et al. 1996; Hwang et al. 2003). PSVs coalesce with incubation time, virtually establishing one large central vacuole. GA hastens this process of vacuolation in aleurone cells, whereas ABA prevents it (Bethke et al. 1999). Because cells pretreated with GA or ABA for 23 hours appeared so morphologically distinct, we examined the effect of NaN_3 on aleurone cells incubated for a shorter time. Figure 1c shows the vacuolated state of aleurone cells incubated in GA or ABA for 12 hours. The cellular morphology of GA-treated aleurone cells were not distinguishable from those treated with ABA (Fig. 1c, d), but NaN_3 susceptibility was still observed only in GA-treated aleurone cells, indicating that GA-induced change in cellular sensitivity to NaN_3 occurs much earlier than coalescence of small PSVs (Fig. 1e).

DNP, an Uncoupler for Oxidative Phosphorylation, Can Also Induce a Rapid Death of GA-preincubated Aleurone Cells, But Not of ABA-pretreated Cells

Inhibition of cytochrome c oxidase by NaN_3 is mediated by forming a complex of azide with the iron of cytochrome c oxidase. Because this metabolic inhibitor also interferes with the action of all different kinds of metal-containing oxidases, we used another kind of inhibitor to determine the effect of inhibition of oxidative phosphorylation on phytohormone pretreated aleurone cells (Beevers 1961). DNP is an uncoupler that prevents the synthesis of ATP through the cytochrome oxidase pathway by dissipating the proton gradient across the mitochondrial cisternae membrane. Aleurone layers were incubated in $CaCl_2$ solution containing GA or ABA for 12 hours and then treated with NaN_3 or DNP for 30 minutes. The aleurone cells were thereafter costained with FDA and FM4-64 to distinguish between live and dead cells. FM4-64 is a lipophilic red-fluorescent dye that rapidly accumulates in dead cells (Fath et al. 2001). Most aleurone cells pretreated with GA for 12 hours were killed either by NaN_3 or DNP, whereas a much smaller fraction of ABA-pretreated cells were affected

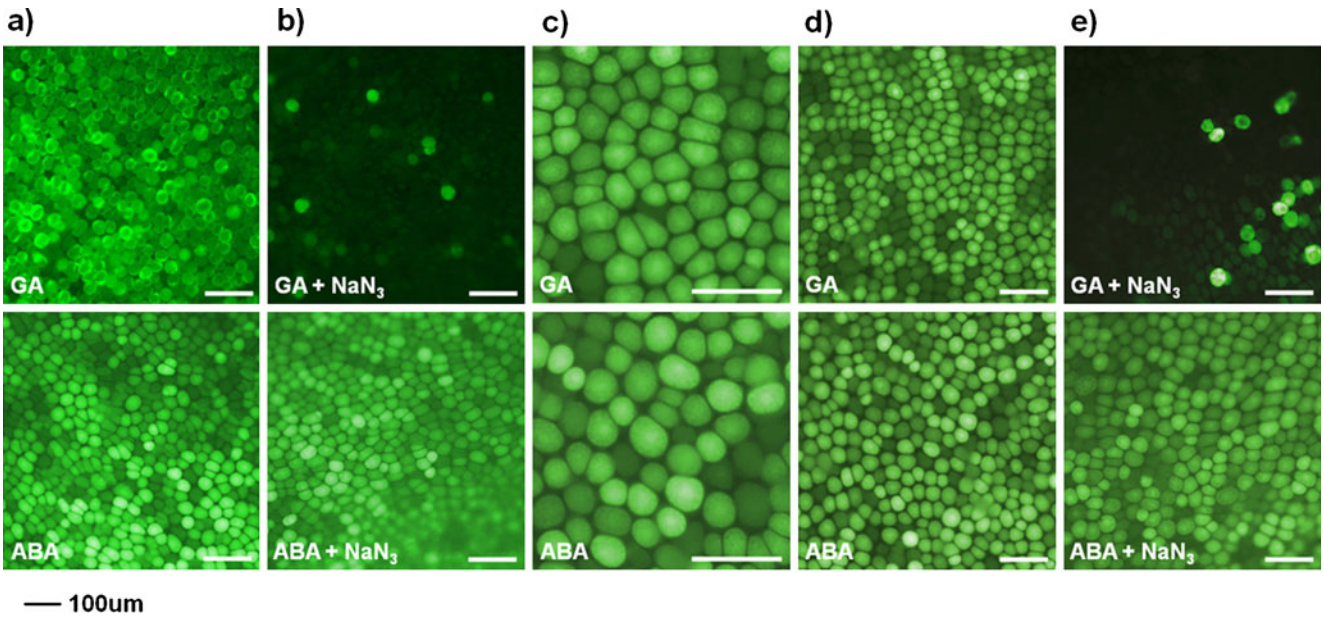

Fig. 1 The oxidative phosphorylation inhibitor NaN_3 can induce rapid death of aleurone cells incubated with GA. FDA staining images of aleurone cells pretreated with GA or ABA for 23 hours (**a**) and then further treated with NaN_3 (**b**) were stained with FDA. Vacuolation of aleurone cells pretreated with GA or ABA for 12 hours (**c**). FDA staining images of aleurone cells pretreated with GA or ABA for 12 hours (**d**) and further treated with NaN_3 (**e**). *Upper* and *lower* panels indicate aleurone layers treated with GA and ABA, respectively. Isolated barley aleurone layers were incubated in 20 mM $CaCl_2$ solution containing GA (10 μM) or ABA (20 μM) for indicated times and further treated with 5 mM NaN_3. After 30 minutes, layers were stained with FDA and examined under fluorescence microscopy to check cell viability. Scale bars in figure represent 100 μM

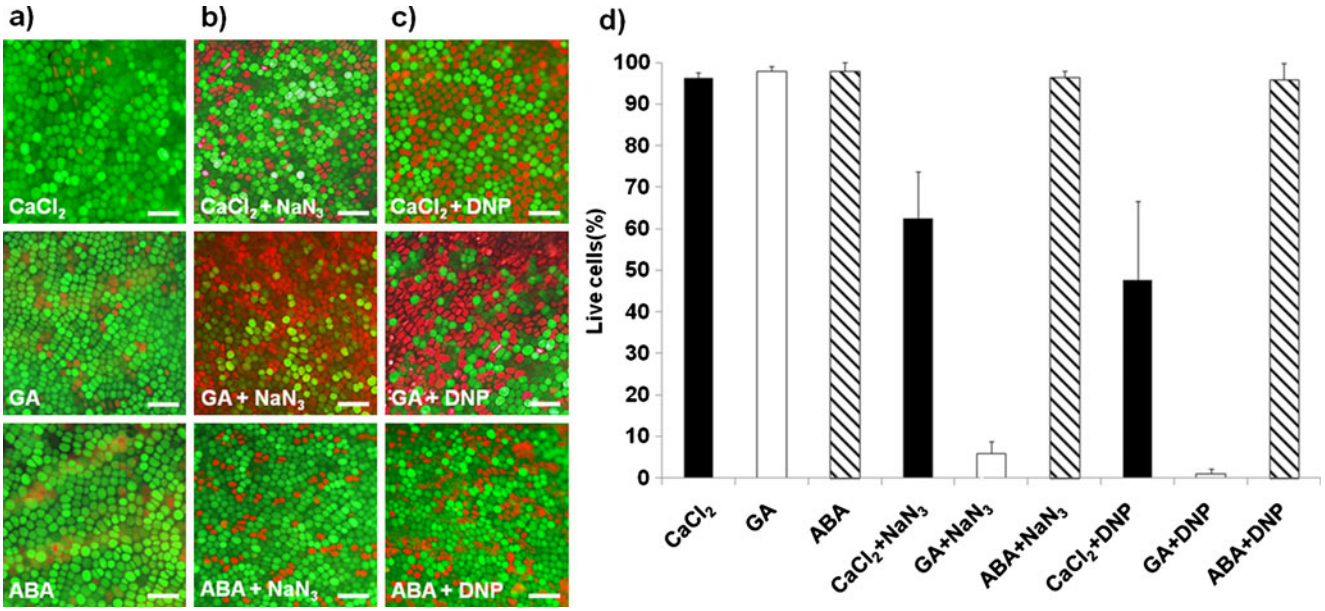

Fig. 2 Oxidative phosphorylation inhibitor DNP can induce rapid death of aleurone cells pretreated with GA. FDA/FM4-64 staining of aleurone cells pretreated with GA or ABA for 12 hours (**a**) and further treated with NaN_3 (**b**) or DNP, 2.5 mM (**c**). **d** Quantification of live (*green*) and dead (*red*) cells from panels (**a**) to (**c**). Numbers of live (*green*) and dead (*red*) cells were counted and expressed as a percentage of the total number of cells captured in the image. Error bars represent standard deviation of the mean (n=3). *Upper*, *middle*, and *lower* panels represent no-hormone ($CaCl_2$ only) and GA- and ABA-treated aleurone layers, respectively. Basic treatments were performed as described in Fig. 1. Scale bars in figure represent 100 μM

(Fig. 2a–c). For example, DNP drove 95 to 99% of GA-pretreated cells to death compared to 40 to 50% cell death in control cells treated with no hormones, whereas less than 5% of cells were affected in ABA-treated cells (Fig. 2d). All these data indicate that GA promotes and ABA decreases the susceptibility of aleurone cells to inhibition of oxidative phosphorylation.

Next, we examined whether anaerobic conditions can mimic the effect of oxidative phosphorylation inhibitors on GA- or ABA-preincubated aleurone cells. The aleurone layers were treated with GA or ABA for 12 hours and then subjected to anoxic conditions. Similar to their response to the metabolic inhibitors, only GA-pretreated cells showed distinct sensitivity to anoxic conditions, in contrast to ABA-treated cells (Fig. 3a–c). However, it took 34 hours for anoxia to induce cell death, which was significantly slower than the metabolic inhibitors, which took approximately 30 minutes (Fig. 3d). This raises the possibility that high metabolic activity may be related to GA-pretreated aleurone cell death induced by oxidative phosphorylation inhibitors. For example, GA is known to stimulate the breakdown of lipid reserves to acetyl CoA through active β-oxidation of fatty acids, which are further oxidized through the TCA cycle. Reduced cofactors such as NADH and FADH, produced during β-oxidation of fatty acid, have to transfer their electrons to oxygen to be recycled. In addition, the oxidative pentose phosphate pathway is known to play an important role in producing NADPH and intermediates for biosynthesis during germination (Buchanan et al. 2000). Anaerobic conditions limit lipid metabolism and the oxidative pentose phosphate pathway, which slows down cellular metabolic activity. Therefore, such a decrease in metabolic activity may allow aleurone cells to survive better under conditions preventing oxidative phosphorylation. Whatever the cause, our data imply that abrupt exposure of barely germinated seeds to extended anoxia, such as under waterlogged conditions, can irreversibly damage aleurone cells and prevent successful post-germinative growth of the seed.

Time Course for GA-induced Change in Cellular State of Aleurone Cells

Aleurone cells have been extensively used as a model system to study the signaling pathways of GA and ABA (Lovegrove & Hooley 2000). GA initiates signaling cascades in cereal aleurone layers, driving the synthesis and secretion of hydrolytic enzymes, most notably α-amylases, whereas ABA antagonizes the effects of GA. During this process, multiple signaling components and events respond in a time-dependent manner. For example, upon addition of GA to barley or wheat aleurone layers, a rapid and transient increase of cytoplasmic Ca^{2+} occurs within 10 minutes (Bush 1996; Gilroy & Jones 1992),

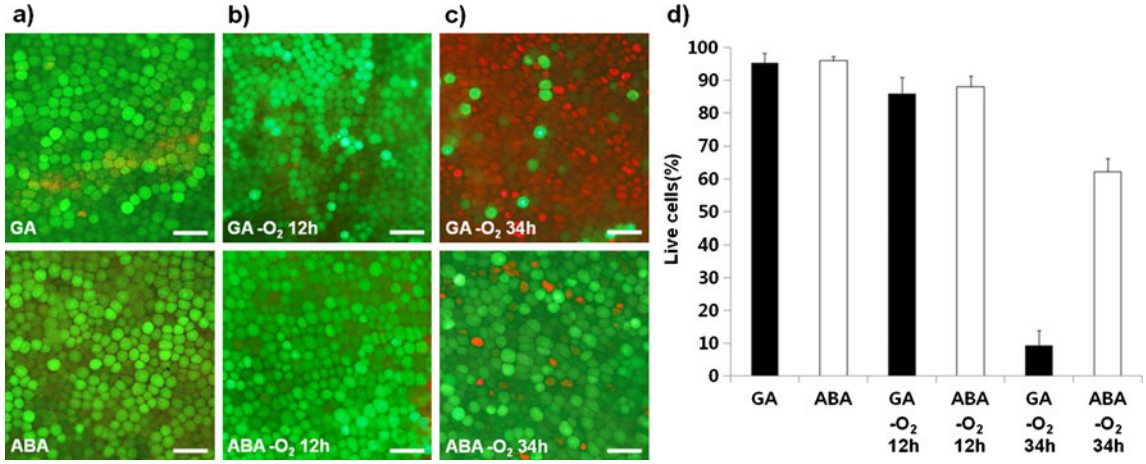

Fig. 3 Anaerobic conditions induce death in GA-pretreated aleurone cells. FDA/FM4-64 staining of aleurone cells pretreated with GA or ABA for 12 hours (**a**) and further treated with anaerobic conditions for 12 hours (**b**) or for 34 hours (**c**). **d** Quantification of live (*green*) and dead (*red*) cells from panels (**a**) to (**c**). Numbers of live (*green*) and dead (*red*) cells were counted and expressed as a percentage of the total number of cells. Error bars represent standard deviation of the mean (n=3). *Upper* and *lower* panels indicate aleurone layers treated with GA and ABA, respectively. Basic treatments were performed as described in Fig. 1. Scale bars in figure represent 100 μM

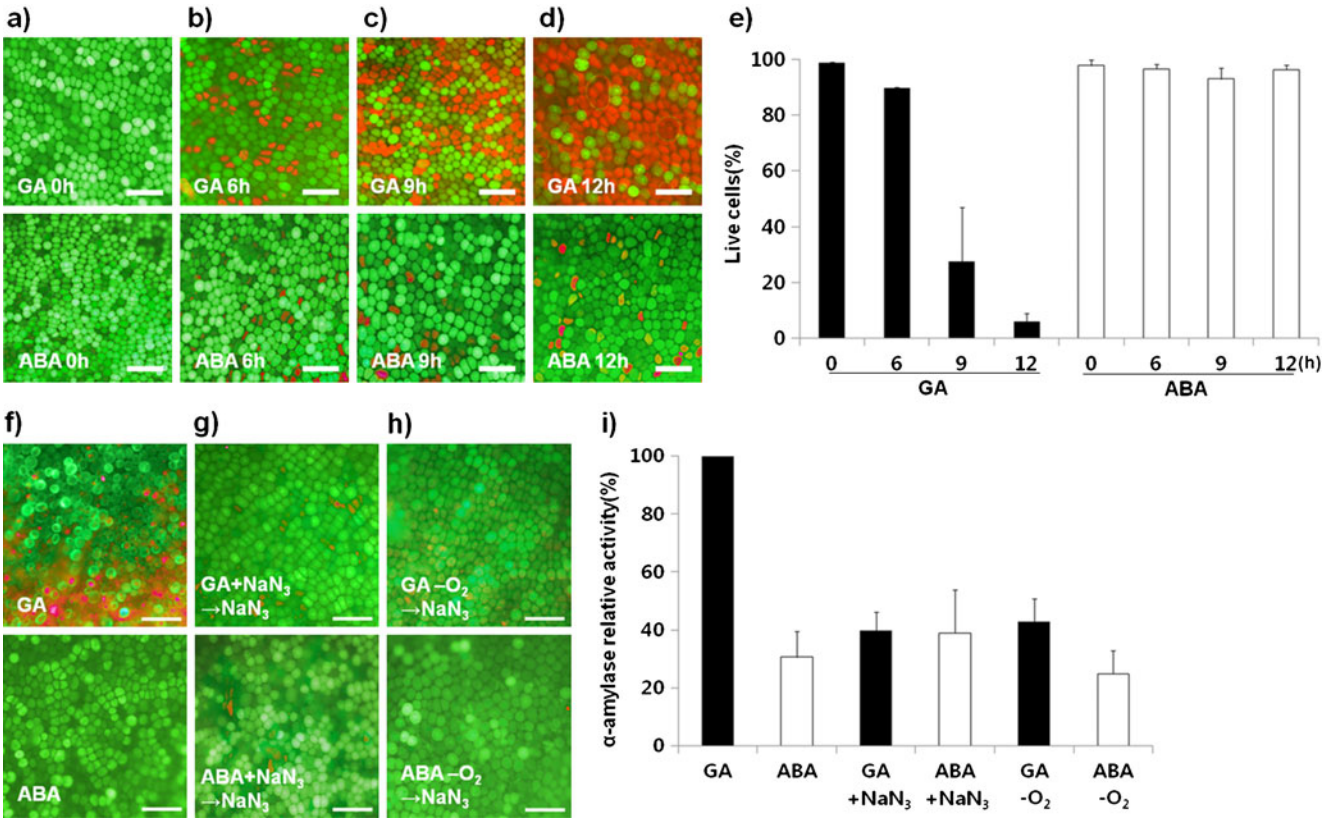

Fig. 4 Time course for induction of sensitivity to oxidative phosphorylation inhibitor in GA pretreated aleurone cells. FDA/FM4-64 staining of aleurone cells pretreated with GA or ABA for indicated times and further treated with NaN_3 (5 mM). Cells pretreated with GA or ABA for 0 hour (**a**), 6 hours (**b**), 9 hours (**c**), and 12 hours (**d**). **e** Quantification of live (*green*) and dead (*red*) cells from panels (**a**) to (**d**). Error bars represent standard deviation of the mean (n=3). FDA/FM4-64 staining of aleurone cells subject to GA treatment simultaneously with no oxidative phosphorylation (**g**) or oxygen deprivation (**h**) for 24 hours then further treated with 5 mM NaN_3 for 30 minutes. Aleurone cells pretreated with GA or ABA under aerobic conditions as controls (**f**). Upper and lower panels indicate aleurone layers treated with GA and ABA, respectively. Basic treatments were performed as described in Fig. 1. Scale bars in figure represent 100 μM

followed by a transient decrease of intracellular pH (Heimovaara-Dijkstra et al. 1994) and a sustained increase in calmodulin (Schuurink et al. 1996). Within 2 hours, cGMP transiently increases (Penson et al. 1996), and GAMyb is induced (Gubler et al. 1995), leading to α-amylase gene expression at around 16 hours (Chandler et al. 1984). Finally, after that, various genes involved in PCD are induced and aleurone cells perished (Brown & Ho 1986; Fath et al. 2001). In this time course of aleurone cell response to GA, we examined how quickly GA can alter the cellular state of aleurone cells to be susceptible to death caused by inhibiting oxidative phosphorylation. Aleurone cells were treated with GA or ABA for 6 to 12 hours and then examined for their susceptibility to the metabolic inhibitor (Fig. 4a–d). Six-hour treatment of GA only subjected 10% of aleurone cells to death by the metabolic inhibitor, but metabolic inhibitor treatment after 9 and 12 hours of incubation with GA caused more than 50 and 90% of cell death, respectively, indicating that GA induces high sensitivity in aleurone cells to inhibition of oxidative phosphorylation within 6 to 12 hours of treatment (Fig. 4e).

Interestingly, when GA or ABA treatment of the cells occurred concurrently with inhibitor or anoxia treatment, we noticed that GA signaling did not seem to proceed. For example, no GA-induced vacuolation (Fig. 4g, h) and no α-amylase synthesis were observed (Fig. 4i). The cells also survived well in the presence of the metabolic inhibitor for extended periods (Fig. 4g, h). Thus, these data suggest that aerobic respiration is required for GA signaling to proceed, and if GA signaling occurs for at least for 6 to 12 hours, the cellular state of the cells changes to be extremely

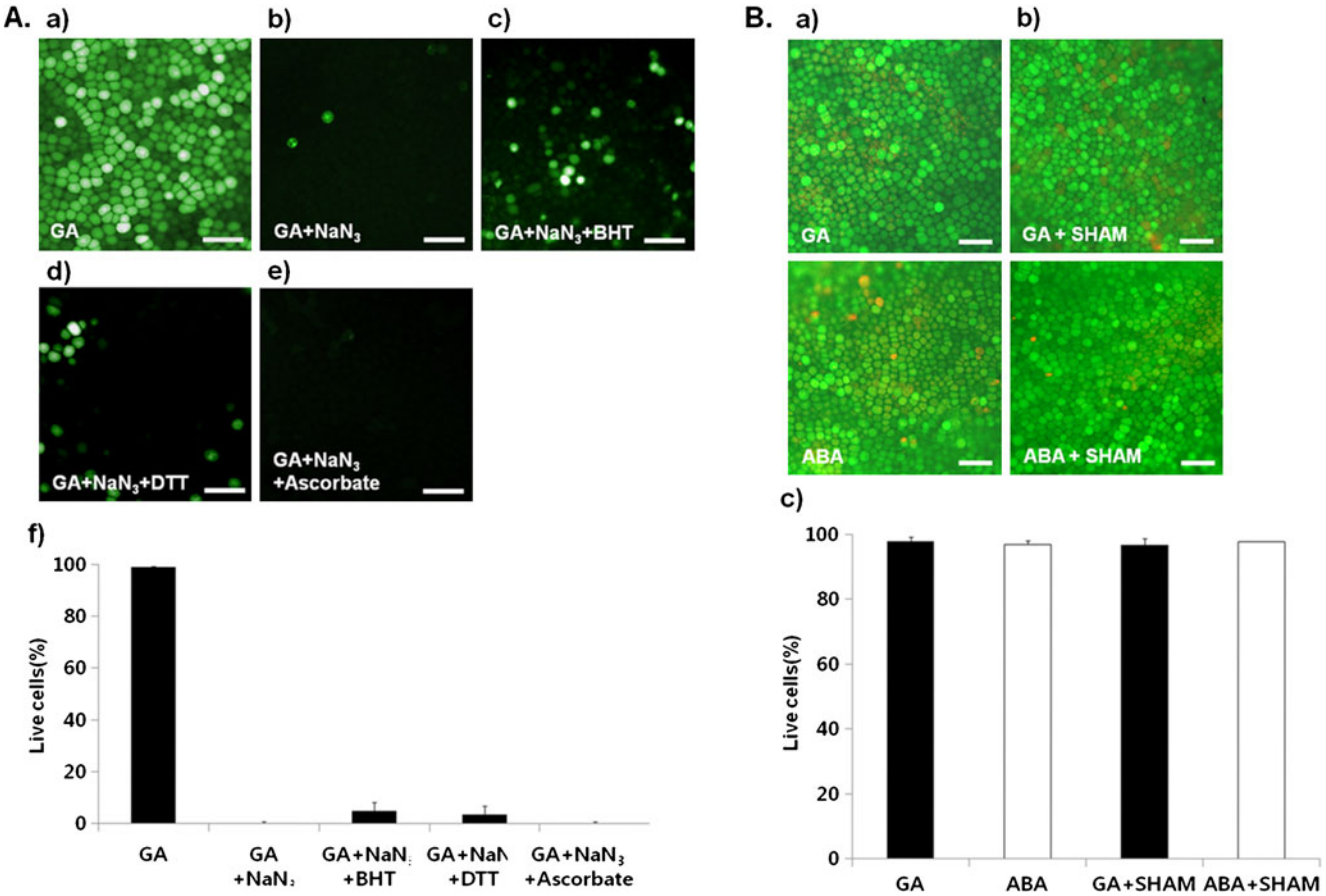

Fig. 5 Responses of phytohormone-pretreated aleurone cells to antioxidants and AOX inhibitor. **A** Antioxidants cannot prevent a rapid cell death induced by inhibition of oxidative phosphorylation. FDA staining of aleurone cells pretreated with GA for 12 hours (**a**) and further treated with 5 mM NaN_3 (**b**). Various antioxidants (BHT, 2 mM; DTT, 20 mM; ascorbate, 100 mM) were co-added with NaN_3 (**c**–**e**). **f** Quantification of live (*green*) and dead (*red*) cells from panels (**a**) to (**e**) of figure **A**. Error bars represent standard deviation of the mean (n=3). Basic treatments were performed as described in Fig. 1. **B** AOX inhibitor does not induce rapid death in GA-pretreated aleurone cells. FDA/FM4-64 staining of aleurone cells pretreated with GA or ABA for 12 hours (**a**) and further treated with 2 mM AOX inhibitor, SHAM (**b**). **c** Quantification of live (*green*) and dead (*red*) cells from panels (**a**) and (**b**) of figure **B**. *Upper* and *lower* panels represent aleurone layers treated with GA and ABA, respectively. The error bars represent standard deviation of the mean (n = 3). Basic treatments were performed as described in Fig. 1. Scale bars in figure represent 100 μM

susceptible to the lethal effects observed from inhibiting oxidative phosphorylation.

Rapid death of aleurone cells induced by oxidative phosphorylation inhibitor is not caused by ROS and GA- or ABA-pretreated aleurone cells do not show any sensitivity to AOX inhibitors in their viability

GA is known to induce PCD in aleurone cells, whereas ABA prevents this process. In wheat aleurone layer, about 85% aleurone cells in the layer were induced to death by 42 hours for GA treatment, whereas only 5% of ABA-treated cells were dead during the same period (Kuo et al. 1996). Previously, ROS were expected to be partly responsible for cell death induced by GA (Bethke & Jones 2001). For example, GA made aleurone cells much more susceptible to ROS like H_2O_2 because it down-regulated the activity of ROS-metabolizing enzymes such as catalase, ascorbate peroxidase, and superoxide dismutase (Fath et al. 2001). GA decreases the transcript levels of catalase after 3 hours of treatment, with superoxide dismutase and ascorbate peroxidase reduced after 12 hours of treatment. Western blot analysis and activity gel assays of ROS-metabolizing enzymes have demonstrated that decreased activities of ascorbate peroxidase and superoxide dismutase occur after 24 hours of GA treatment, however, catalase activity was significantly reduced even after 12 hours (Fath et al. 2002). We examined whether ROS are involved in the rapid cell death induced by oxidative phosphorylation inhibitors. We tested whether antioxidants like ascorbate and BHT are able to ameliorate the detrimental effects of NaN_3 on GA-pretreated aleurone cells. As indicated in Fig. 5a, rapid cell death, induced by oxidative phosphorylation inhibitor, was not at all affected by cotreatment with antioxidants, indicating that ROS themselves are not the direct cause of rapid death.

Because the inhibition of cytochrome oxidase pathway rapidly kills the GA-pretreated cells, we first examined whether the viability of cells pretreated with GA or ABA is also affected by inhibition of AOX pathway, an another component of plant mitochondrial respiration, which does not involve any oxidative phosphorylation. Figure 5b shows the sensitivity of GA- or ABA-pretreated aleurone cells to an AOX inhibitor. Unlike oxidative phosphorylation inhibitors, the AOX inhibitor did not affect the viability of phytohormone pretreated cells (Fig. 5b). Therefore, GA-pretreatment rendered aleurone cells extremely sensitive only to inhibition of oxidative phosphorylation.

To date, GA has been known to induce a series of sequential cellular events, including cytoplasmic Ca^{2+} fluctuation, transient cGMP increase, calmodulin induction, vacuolar acidification, vacuolar fusion, GAMyb induction, and α-amylase expression, and so on. In this study, we provide another new marker for cellular response to GA: GA-induced change in the cellular sensitivity for inhibition of oxidative phosphorylation. It still remains undetermined how inhibition of oxidative phosphorylation induces rapid death of GA-pretreated aleurone cells, whereas ABA-treated cells remain unaffected. The phytohormone GA may partition all the electrons produced during mitochondrial respiration only to the cytochrome oxidase pathway. As a result, freezing this pathway may lead to complete inhibition of recycling of reducing power, with deleterious consequences. Both of increase in ROS production and decrease in ROS scavenging activity would contribute to the PCD of aleurone cells induced by GA. Our data implicate that GA would drive aleurone cells to respire more through the cytochrome oxidase pathway, which would produce more ROS. In the future, we need to examine whether the cellular change in sensitivity to oxidative phosphorylation inhibitors actually accompanies an increase in ROS production.

Acknowledgments The research described in this paper was supported by the Basic Science Research Program through the National Research Foundation of Korea (331-2007-1-C00226; 2008-0058877; 2009-0076503).

We would like to thank Dr. Lim, Jun; Dr. Shim, Yhong-Hee; and Dr. Kawasaki, Ichiro at the Department of Bioscience and Biotechnology at Konkuk University for sharing their experimental facility, including the fluorescence microscope.

References

Beevers H (1961) Respiratory metabolism in plants. Row, Peterson and Company, Whilte Plains, New York

Bethke PC, Fath A, Spiegel YN, Hwang Y-S, Jones RL (2002) Abscisic acid, gibberellin and cell viability in cereal aleurone. Euphytica 126:3–11

Bethke PC, Swanson SJ, Hilmer S, Jones RL (1998) From storage compartment to lytic organelle: the metamorphosis of the aleurone protein storage vacuole. Ann Bot 82:399–412

Bethke PC, Hillmer S, Jones RL (1996) Isolation of intact protein storage vacuoles from barley aleurone (Identification of aspartic and cysteine proteases). Plant Physiol 110:521–529

Bethke PC, Jones RL (2001) Cell death of barley aleurone protoplasts is mediated by reactive oxygen species. Plant J 25:19–29

Bethke PC, Lonsdale JE, Fath A, Jones RL (1999) Hormonally regulated programmed cell death in barley aleurone cells. Plant Cell 11:1033–1046

Botha FC, Potgieter GP, Botha A-M (1992) Respiratory metabolism and gene expression during seed germination. Plant Growth Regul 11:211–224

Boyd V, Cholewa OM, Papas KK (2008) Limitations in the use of fluorescein diacetate/propidium iodide (FDA/PI) and cell permeable nucleic acid stains for viability measurements of isolated islets of Langerhans. Curr Trends Biotechnol Pharm 2:286–304

Brown PH, Ho T-H (1986) Barley aleurone layers secrete a nuclease in response to gibberellic acid. Plant Physiol 82:801–806

Buchanan BB, Gruissem W, Jones RL (2000) Biochemistry and molecular biology of plants. American society of plant physiologists, Rockville

Bush DS (1996) Effects of gibberellic acid and environmental factors on cytosolic calcium in wheat aleurone cells. Planta 199:89–99

Chandler PM, Zwar JA, Jacobsen JV, Higgins TJV, Inglis AS (1984) The effect of gibberellic acid and abscisic acid on α-amylase mRNA levels in barley aleurone layers: studies using an α-amylase cDNA clone. Plant Mol Biol 3:407–418

Corpas FJ, Barroso JB, del Rio LA (2001) Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. Trends Plant Sci 6:145–150

Esashi Y, Sakai Y, Ushizawa R (1981) Cyanide-sensitive and cyanide-resistant respiration in the germination of cocklebur seeds. Plant Physiol 67:503–508

Fath A, Bethke PC, Beligni V, Jones RL (2002) Active oxygen and cell death in cereal aleurone cells. J Exp Bot 53:1273–1282

Fath A, Bethke PC, Lonsdale J, Meza-Romero R, Jones RL (2000) Programmed cell death in cereal aleurone. Plant Mol Biol 44:255–266

Fath A, Bethke PC, Jones RL (1999) Barley aleurone cell death is not apoptotic: characterization of nuclease activities and DNA degradation. Plant J 20:305–315

Fath A, Bethke PC, Jones RL (2001) Enzymes that scavenge reactive oxygen species are downregulated prior to gibberellic acid-induced programmed cell death in barley aleurone. Plant Physiol 126:156–166

Fleury C, Mignotte B, Vayssiere JL (2002) Mitochondrial reactive oxygen species in cell death signaling. Biochimie 84:131–141

Gilroy S, Jones RL (1992) Gibberellic acid and abscisic acid coordinately regulate cytoplasmic calcium and secretory activity in barley aleurone protoplasts. Proc Natl Acad Sci U S A 89:3591–3595

Gubler F, Kalla R, Roberts JK, Jacobsen JV (1995) Gibberellin-regulated expression of a myb gene in barley aleurone cells: evidence for Myb transactviation of high-pI α-amylase gene promoter. Plant Cell 7:1879–1891

Heimovaara-Dijkstra S, Heistek JC, Wang M (1994) Counteractive effects of ABA and GA_3 on extracellular and intracellular pH and malate in barley aleurone. Plant Physiol 106:359–365

Hwang YS, Bethke PC, Gubler F, Jones RL (2003) cPrG-HCl a potential H^+/Cl^- symporter prevents acidification of storage vacuoles in aleurone cells and inhibits GA-dependent hydrolysis of storage protein and phytate. Plant J 35:154–163

Jones KH, Senft JA (1985) An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. J Histochem Cytochem 33:77–79

Jones RL (1967) The bioassay of gibberellins. Planta 72:155–161

Jones RL (1969) The fine structure of barley aleurone cells. Planta 85:359–374

Juszczuk IM, Rychter AM (2003) Alternative oxidase in higher plants. Acta Biochim Pol 50:1257–1271

Kuo A, Cappelluti S, Cervantes-Cervantes M, Rodriguez M, Bush DS (1996) Okadaic acid, a protein phosphatase inhibitor, blocks calcium changes, gene expression, and cell death induced by gibberellin in wheat aleurone cells. Plant Cell 8:259–269

Lovegrove A, Hooley R (2000) Gibberellin and abscisic acid signalling in aleurone. Trends Plant Sci 5:102–110

Møller IM (2001) Plant mitochrondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annu Rev Plant Physiol Plant Mol Biol 52:561–591

Maxwell DP, Wang Y, McIntosh L (1999) The alternative oxidase lowers mitochondrial reactive oxygen production in plant cells. Proc Natl Acad Sci U S A 96:8271–8276

Mori IC, Muraya Y, Uraji M (2009) Integration of ROS and hormone signaling reactive oxygen species in plant signaling. Springer, Berlin Heidelberg

Penson SP, Schuurink RC, Fath A, Gubler F, Jacobsen JV, Jones RL (1996) cGMP is required for gibberellic acid-induced gene expression in barley aleurone. Plant Cell 8:2325–2333

Popov VN, Simonian RA, Skulachev VP, Starkov AA (1997) Inhibition of the alternative oxidase stimulates H2O2 production in plant mitochondria. FEBS Lett 415:87–90

Puntarulo S, Sanchez RA, Boveris A (1988) Hydrogen peroxide metabolism in soybean embryonic axes at the onset of germination. Plant Physiol 86:626–630

Purvis AC (1997) Role of the alternative oxidase in limiting superoxide production by plant mitochondria. Physiol Plant 100:165–170

Ritchie S, Swanson SJ, Gilroy S (2000) Physiology of the aleurone layer and starchy endosperm during grain development and early seedling growth: New insights from cell and molecular biology. Seed Sci Res 10:193–212

Schuurink RC, Chan PV, Jones RL (1996) Modulation of calmodulin mRNA and protein levels in barley aleurone. Plant Physiol 111:371–380

Swanson SJ, Jones RL (1996) Gibberellic acid induces vacuolar acidification in barley aleurone. Plant Cell 8:2211–2221

Vanlerberghe GC, McIntosh L (1997) Althernative oxidase: from gene to function. Annu Rev Plant Physiol Plant Mol Biol 48:703–734

Wagner AM, Moore AL (1997) Structure and function of the plant alternative oxidase: its putative role in the oxygen defence mechanism. Biosci Rep 17:319–333

Yentur S, Leopold AC (1976) Respiratory transition during seed germination. Plant Physiol 57:274–276