Rapid determination of seed vigor based on the level of superoxide generation during early imbibition

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It has been reported that a large amount of reactive oxygen species (ROS) is produced during seed imbibition and this ROS is related to seed vigor. To make this physiological mechanism clear, we have used 2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo(1,2-α)pyrazin-3-one (MCLA) as a sensitive and physiologically compatible probe for the determination of superoxide anion ($O_2^{\bullet-}$) production *in vivo.* Our results showed that dry rice (*Oryzae sativa* L.) seed embryo cells possessed the capacity to generate $O_2^{\bullet-}$. Conversely, the $O_2^{\bullet-}$ production of seed embryo cells was inhibited by quinacrine (QA) and diphenylene iodonium (DPI), two specific inhibitors of NADPH oxidase, and $O_2^{\bullet-}$ induced MCLA-mediated chemiluminescence was also blocked by superoxide dismutase (SOD). Additionally, $O_2^{\bullet-}$-production ability increased dramatically in a NADPH-dependent way in the plasma membrane protein abstract from rice seed embryo cells, whereas SOD and the inhibitors mentioned above suppressed $O_2^{\bullet-}$ production. These preliminary results suggested that rice seeds contained intrinsic NADPH oxidase activity. To validate this conclusion, dichlorofluorescein (DCF) fluorescence staining was used (observed under a laser scanning microscope, LSM) to reflect the *in situ* assessment of $O_2^{\bullet-}$-generation. The position of $O_2^{\bullet-}$ production located at the plasma membrane. Additionally the ability to synthesize $O_2^{\bullet-}$ was activated directly by calcium ions. These observations are in accord with the character of NADPH oxidase catalyzed $O_2^{\bullet-}$-generation. All these results indicated that NADPH oxidase contribute to $O_2^{\bullet-}$ production and release to the outside. We concluded that NADPH oxidase plays an intrinsic role as an NADPH sensor, so, measuring the $O_2^{\bullet-}$ one can monitor the NADPH concentration, which is an index of seed vigor. Therefore the $O_2^{\bullet-}$ generation during early imbibition can serve as a rapid measurement of seed vigor.

Introduction

More and more experiments have demonstrated that ROS can promote seed vigor.[1] It has been reported that the production of ROS during seed germination in fact represents an active, beneficial biological reaction that is associated with high germination capacity and vigorous seedling development.[2] There are several evidence: *e.g.* H_2O_2 promotes seed germination by the oxidated decomposition of the germination inhibitor(s) present in the pericarp.[1] ROS production by germinating seeds represents an active, development control physiological function, protecting the emerging seedling against pathogen attack.[2] ROS can function as cellular second messengers that are likely to modulate many different proteins, leading to a variety of responses.[3] However, an enzymatic dismutation step must firstly take place to produce from the $O_2^{\bullet-}$ the more stable H_2O_2 derivative that is required for a viable long-range cell-to-cell signal or for passing membranes.[4] It is possible that there is an endogenous mechanism to generate ROS in dry seed. During rice seed aging, this kind of ability declines until it vanishes, and adding extrinsic H_2O_2 can resume the seed vigor.[1]

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In recent years, more and more studies have demonstrated that NADPH oxidase contribute to the production of ROS. NADPH oxidase widely exists in animals, plants, and filamentous fungi.[5] In plants, the NADPH oxidase homologs have been named respiratory burst oxidase homologs (Rboh),[6,7] and NADPH oxidase contains cytosolic FAD- and NADPH-binding domains and six conserved transmembrane helices. The third and fifth bind two heme groups through four critical His residues. The heme groups are required for transfer of electrons across the membrane to oxygen, the extracellular (EC) acceptor, to generate $O_2^{\bullet-}$.[8,9] The N-terminal region elongation factor (EF) hands juxtaposed to the C-terminal end indicate an interaction by which calcium-dependent activity is regulated.[10] $O_2^{\bullet-}$ can subsequently be dismutated to H_2O_2 spontaneously or in a reaction catalyzed by superoxide dismutase.[2] Recent studies have focused on ROS generation through the intrinsic molecular properties of NADPH oxidase as they are related to their function in plants.[11–13]

Proteins with a sequence homologous to the NADPH oxidase gene have been discovered in rice,[14,15] but whether dry rice seeds possess intrinsic NADPH oxidase activity is unclear. In this work, we have attempted to reveal the correlation mechanism of the ROS generation during the early imbibition and seed vigor. We observed the ROS generation ranging from intact seed, embryo cells to plasma membrane protein abstract. Combined with the NADPH oxidase inhibitor and ROS scavenging experiments it showed that there existed intrinsic NADPH oxidase in rice seeds.

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The $O_2^{\bullet-}$ generated by NADPH oxidase is released to the outside of embryo cells and can be measured using the MCLA-mediated chemiluminescence technique with the a low noise and high-sensitivity single photon counting (SPC) device. Overall, these results showed that NADPH oxidase plays a role as an intrinsic NADPH sensor, according to which we have developed a facilely measurable index to measure NADPH concentration which is an important index of seed vigor.

Materials and methods

Plant material

Rice seeds of *Oryza sativa* L. strain 8072-2 were obtained from Guangdong Academy of Agricultural Sciences. They were harvested in 2001, 2003, 2004, 2005 and 2006 respectively. All the samples were packed in separate cloth bags, stored in a desiccator with silica gel and kept at room temperature (15–28 °C). Seeds in all experiments were selected and the embryos were isolated from the seeds carefully. The bottom part of the seed embryos were cut to ground using a pestle and mortar in a phosphate buffer containing 3 mM $CaCl_2$. The homogenate was filtered through a 50 μm nylon filter, and the resulting filtrate was centrifuged at 1290 rev min^{-1} (585 × *g*) for 5 min, the supernatant was discarded and the above process was repeated two times. The collected seed embryo cells were suspended in PBS.

Reagents

2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo(1,2-α)pyrazin-3-one (MCLA) was obtained from Kasei kogyo (Tokyo, Japan); 2′,7′-dihydrodichlorofluorescein diacetate (H_2DCF-DA), polyethylene glycol 3350 (PEG 3350), Triton X-100, superoxide dismutase (SOD) leupeptin, pepstatin, diphenylene iodonium (DPI) 2,3,5-triphenyltetrazoliumchloride (TTC) and $NADPHNa_4$ were from Sigma (St. Louis, MO63178, USA); Aprotinin were from Amers, Dextran T-500 was obtained from Pharmacia, dithiothreitol (DTT) and Tris were obtained from Promega, phenylmethylsulfonylfluoride (PMSF) and [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES) were obtained from Biomol; polyvinylpyrrolidone (PVP) was obtained from BASF. All other chemicals were purchased from Guangzhou Zhangchen Chemical Co. (Guangzhou, China).

Preparation and fractionation of membranes

The seed embryo cells were fragmented using a sonicator programmable ultrasonic processor in a buffer (15 μL mg^{-1} rice seed embryos) containing 0.25 M sucrose (Suc), 50 mM HEPES–KOH (pH 7.2), 3 mM EDTA, 1 mM dithiothreitol (DTT), 0.6% (w/v) polyvinylpyrrolidone, 3.6 mM L-Cys, 0.1 mM $MgCl_2$, and a cocktail of protease inhibitors including phenylmethylsulfonyl fluoride (2 mM), aprotinin (10 μg ml^{-1}), leupeptin (10 μg ml^{-1}), and pepstatin (10 μg ml^{-1}). The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA), and the resulting filtrate was centrifuged at 10000 *g* for 45 min. Microsomal membranes were pelleted from the supernatant by centrifugation at 203000 *g* for 60 min.

For aqueous two-phase partitioning, the microsomes from rice seed embryos were gently resuspended in 0.33 M Suc and 5 mM potassium phosphate (pH 7.8), 3 mM KCl, and protease inhibitors. The suspension was then fractionated by the aqueous two-phase partitioning method according to the batch procedure as described.[16] Phase separations were carried out in a series of 10 g phase systems with a final composition of 6.2% (w/w) dextran T-500, 6.2% (w/w) polyethylene glycol 3350, 0.33 M Suc, and 5 mM potassium phosphate (pH 7.8), 3 mM KCl, and protease inhibitors. Three successive rounds of partitioning yielded final upper phases (U3 and U3′) and a lower phase (L3), The combined upper phase was enriched in plasma membrane vesicles and the lower phase contained intracellular membranes. The final upper and lower phases were diluted 5- and 10-fold, respectively, in ice-cold Tris–HCl dilution buffer (10 mM, pH 7.4) containing 0.25 M Suc, 3 mM EDTA, 1 mM DTT, 3.6 mM L-Cys, 0.1 mM $MgCl_2$, and the protease inhibitors. The fractions were centrifuged at 203000 *g* for 60 min. The pellets were then resuspended in Tris–HCl dilution buffer and used immediately for further analysis. All procedures were carried out at 4 °C.

Determination of $O_2^{\bullet-}$ release

Using MCLA for the determination of $O_2^{\bullet-}$ production *in vitro*, the intact rice SWC (seed without capsule 5 seeds ml^{-1}) was mixed with MCLA (2 μM) with or without SOD and the NADPH oxidase inhibitors DPI (50 μM) and QA (100 μM). MCLA-mediated chemiluminescence of the mixture was detected with a low noise and highly sensitive single photon counting (SPC) device developed by our lab. The system consists of a temperature-controlled light-tight sample chamber, a single photon counting photomultiplier tube (PMT; MP962, Perkin, Elmer optoelectronics, Wiesbaden, Germany) and a computer-controlled photon counter module. Rice seeds of equal amount were weighed, put in a quartz cuvette and kept in sample ponds of the dark box for darkening for 20–30 min, in order to avoid photo-induced delayed luminescence. The acquisition time of each experiment was about 10 min considering the single photon of luminescence collected every 1 s as a datum. The mean of these data served as a measurement. The chemiluminescence (CL) intensity was normalized to cps g^{-1} dry weight (cps g^{-1} dw).

Embryo cells isolated from rice seeds of different age, respectively harvested in 2001, 2002, 2004, 2005 and 2006, which induced MCLA-mediated CL, were also measured. All of these operations were made at room temperature (approximately 25 °C).

Another fluorescence probe, H_2DCF-DA, was used to observe the ROS formation in suspending solution of rice seed embryo cells under the luminescence spectrometer (LS55, Perkin-Elmer, UK). The seed embryo cells (SEC 10^6 cells ml^{-1}) were mixed with H_2DCF-DA (2 μM) with or without catalase (100 μg mL^{-1}) and the NADPH oxidase inhibitors DPI (50 μM) and QA (100 μM). The mixture was measured immediately using the luminescence spectrometer, which was set to an excitation wavelength of 488 nm and emission wavelength of 525 nm, with a slit width of 2.5 nm. All of these operations were made at room temperature (approximately 25 °C).

Plasma membranes were isolated and purified from the rice seed embryo cells using a two-phase partitioning system as described.[6,17] The protein content of plasma membranes was determined by the method of Bradford (1976). The purified plasma membrane preparation was verified by using specific inhibitors

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of marker enzymes.[17] The NADPH oxidase activity of the plasma membrane was determined based on the rate of $O_2^{\bullet-}$ formation detected *via* MCLA-mediated chemiluminescence. Rice seed embryo extract proteins (10 μg) were mixed with MCLA (2 mM) and with or without NADPH. The MCLA-mediated chemiluminescence of the mixtures in the measurement solution (5 mM Ca^{2+}) was measured by SPC. The activity of NADPH oxidase was expressed as cps μg^{-1} protein. In inhibitor and ROS scavenging experiments, DPI (50 μM), quinacrine (100 μM) and SOD (50 U mL^{-1}) were added 30 min prior to measurement.

Heat-inactivation treatment

1 mL samples (rice seed without capsule (SWC) or seed embryo cell plasma membranes were put into a 1.5 mL Eppendorf tube, and then water bathed at 100 °C for 2 h.

ROS-synthesis *in situ* assays

The seed embryo cells (SEC 10^6 cells ml^{-1}) were mixed with H_2DCF-DA (2 μM) and with or without the NADPH oxidase inhibitors DPI (50 μM) and QA (100 μM). The DCF fluorescence staining was performed using a commercial laser scanning microscope (LSM510/ConfoCor2) combination system (Zeiss, Jena, Germany). For excitation, a green-ion laser (488 nm) set on 3% power was used, the emission fluorescence was collected by the 500–550 nm bandpass. The viability of the embryo cell under the PBS was >80%, as tested by fluorescence diacetate (FDA) staining.

Calcium-dependent $O_2^{\bullet-}$ production

NADPH oxidase was assayed in membranes by a modified assay based on chemiluminescence of MCLA induced by $O_2^{\bullet-}$. The assay reaction medium contained 10 μg upper phase proteins, 2 mM MCLA, and 0.15 mM NADPH in 1 mL 50 mM Tris–HCl buffer (pH 7.4) with varying concentration of $CaCl_2$ (0–10 mM) or 10 mM ethylene glycol bis(2-aminoethyl)tetraacetic acid (EGTA). The reaction was initiated with the addition of NADPH. MCLA chemiluminescence was determined by SPC.

TTC reduction test

1 mL seed embryo cell suspension, 1 mL 0.8% (w/v) TTC and 4 mL 0.5 M Na_2HPO_4–KH_2PO_4 (pH 7.4) were put into a 15 mL centrifuge tube with a screw cap. The contents of the tubes were mixed using a vortex mixer for 30 s and incubated at 35 °C for 2 h, then 2 mL acetoacetate was added to each tube. The tubes were shaken using the vortex mixer and then centrifuged at 2150 rev min^{-1} (975 × *g*) for 20 min. The suspension was transferred to a test tube. One more extraction of the retained seed embryo cells with 2 mL acetoacetate was done (further extraction did not provide any red color). The supernatant was transferred to the test tube and the combined suspension was shaken. Finally, the optical density of the combined suspension was measured at 485 nm using a spectrophotometer (Lambda 35, UV-VIS spectrometer, Perkin-Elmer America). The control sample was used to zero the spectrophotometer. The triphenyl formazan (TF) concentration was then obtained from the standard curve. Rice seeds harvested in 2001, 2002, 2004, 2005 and 2006 were measured.

Results

NADPH oxidase *in vivo* assays

Cells from different plant species contain oxidases that generate ROS and regulate vital physiological functions such as seed germination and root hair growth.[18,19] We reasoned by analogy that ROS required for seed germination was probably generated by these oxidases during early imbibition. We used the MCLA-mediated chemiluminescence assay to determine whether the intact rice seed without capsule (SWC) generates $O_2^{\bullet-}$. The MCLA specially reacts with $O_2^{\bullet-}$ to generate chemiluminescence. As expected, the SWC let MCLA generate chemiluminescence, however, the heat-inactivated SWC (SWC^H) failed to produce the MCLA-mediated chemiluminescence. Furthermore, NADPH oxidase inhibitors DPI (50 μM) and QA (100 μM) obviously abated this kind of chemiluminescence respectively by 78% and 73% (Fig. 1). The ability of SWC induced MCLA-mediated chemiluminescence was also blocked by SOD. These observations initially suggested that there existed NADPH oxidase activity in rice seed, and this was the main contributor to ROS production.

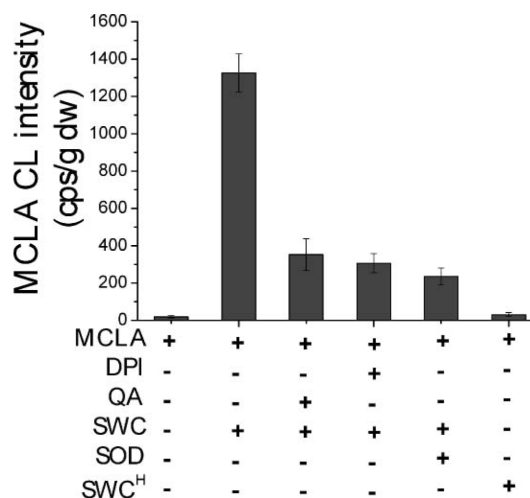

Fig. 1 Superoxide reacted with MCLA to generate chemiluminescence in the presence (+) or absence (−) of SWC; SWC^H was also tested. SWC induced MCLA-mediated chemiluminescence is inhibited by SOD and NADPH oxidase inhibitors DPI and QA. Data are mean ±SE of four repeats and represent one of three different experiments that yielded similar results. SWC: seed without capsule; SWC^H: heat-inactivated SWC.

NADPH oxidase *in vitro* assays

Seed germination is accompanied by a steep rise in ROS release originating from the embryo as well as the seed coat.[2] Embryo cells were isolated from the rice seed as described in Materials and methods. The ability of the rice seed embryo cell (SEC) to oxidize redox-sensitive 2′,7′-dihydro-dichlorofluorescein diacetate (H_2DCF-DA) to fluorescecent dichlorofluorescein (DCF) was also studied. Results showed that the SEC converted H_2DCF-DA into DCF, but heat-inactivated SEC (SEC^H) failed to do so. Furthermore, the ability of SEC to oxidize H_2DCF-DA into DCF was blocked by hydrogen peroxide scavenger catalase (100 μg mL^{-1}) and NADPH oxidase inhibitors DPI (50 μM) and QA

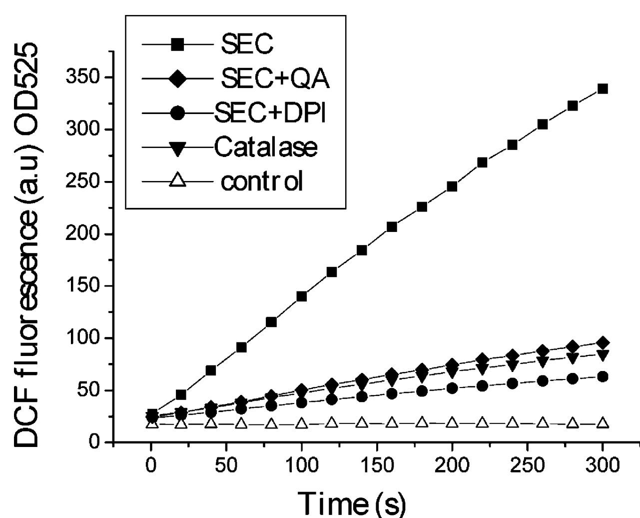

Fig. 2 Kinetics of ROS generation determined by DCF fluorescence assay. Shown are SEC (filled squares), SEC plus QA (filled diamond), SEC plus DPI (filled circle), SEC plus catalase (filled triangle), and the control which only contains H_2DCF-DA (open triangle). SEC: seed embryo cell.

(100 μM) (Fig. 2). These observations suggested that NADPH oxidase was present in rice seed embryo cells.

NADPH oxidase is required for transfer of electrons across the membrane to oxygen, the extracellular (EC) acceptor, to generate superoxide anion.[10] To further identify whether the rice seed embryo cells possess intrinsic NADPH oxidase activity the plasma membranes were isolated and purified with a two-phase partitioning system as described in Materials and methods. NADPH oxidase activity of the reconstruct system *in vitro* was measured using the MCLA-mediated chemiluminescence technique. Adding NADPH to a seed embryo cell plasma membrane (SPM) solution boosted its ability to generate MCLA-mediated chemiluminescence. In contrast, heat-inactivated SPM (SPM^H) failed to activate MCLA. The ability of SPM to generate MCLA chemiluminescence was blocked by superoxide anion scavenger SOD and the NADPH oxidase inhibitors DPI and QA (Fig. 3). Based on above results, we can conclude that ROS originate mainly from plasma membrane (PM) NADPH oxidase.

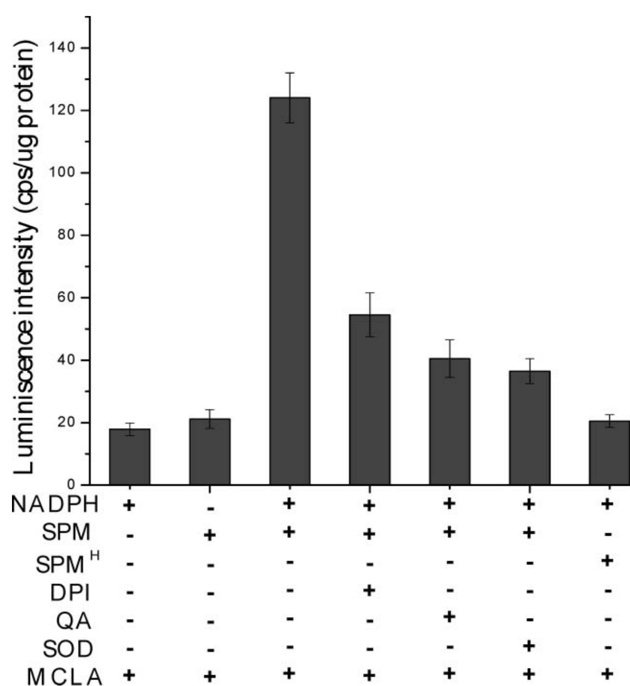

Fig. 3 Cell-free reconstitution of the $O_2^{\bullet -}$ generation system. The generation of $O_2^{\bullet -}$ by purified SPM of isolated rice seed embryo cells was measured by the $O_2^{\bullet -}$ induced chemiluminescence of MCLA in the presence and absence of SOD, NADPH oxidase inhibitors (DPI and QA) and NADPH, as indicated. SOD, DPI and QA were added 30 min before the reaction was started by the addition of NADPH. Also the heat-inactivated SPM served as a control. SPM: seed embryo cell plasma membranes; SPM^H: heat-inactivated SPM. Data are mean ±SE of five repeats and represent one of four different experiments that yielded similar results.

NADPH oxidase *in situ* assays

To validate the presence of NADPH oxidase in rice seed embryo cells, we performed *in situ* H_2DCF-DA assays on isolated seed embryo cells. H_2DCF-DA was oxidized into DCF fluorescence by ROS, and the DCF fluorescence accumulated where ROS was generated. The green fluorescence indicated the oxidation of H_2DCF-DA to DCF fluorescence *in situ* of ROS production. In this experiment, H_2DCF-DA was used to detect the position of ROS production in rice seed embryo cells. The real-time monitoring of ROS production was performed under a laser scanning microscope (LSM), and the DCF fluorescence was imaged (Fig. 4). DCF fluorescence firstly emerged at the position of the plasma membrane and increased rapidly (Fig. 4(A), (B) and (C)). Our result was in agreement with the notion that ROS production was localized at the plasma membrane and could be attributed to NADPH oxidase catalyzed. In contrast, this kind of fluorescence was not observed in heat-inactivated seed embryo cells (SEC^H) and in SEC when DPI or QA were added (data not shown).

Activation of NADPH oxidase activity by calcium ions

The plant NADPH oxidase homologs contain two additional N-terminal diversification of calcium-binding elongation factor (EF) hands which can bind Ca^{2+} and consequently activate the NADPH oxidase.[10,19] In our experiment, the possible interaction of NADPH oxidase and Ca^{2+} was examined in two-phase fractionated embryo cell extracts of rice seed. NADPH oxidase showed high basal activity without Ca^{2+} added to the reaction medium (Fig. 5). The addition of 10 mM EGTA did not influence the basal level of $O_2^{\bullet -}$ production. The activity was further induced by the addition of Ca^{2+} starting from 30 mM to a maximum 2-fold enhancement at millimolar levels of Ca^{2+}. The induction by calcium was blocked by the addition of EGTA (Fig. 5). The result was agreed with the notion that the progress of NADPH oxidase catalyzed superoxide anion production is activated directly by calcium ions.

Relationship between ROS production and reductive hydrogen

According to the principle of seed vigor fast assessing (TTC-test), TTC can be reduced by reductive hydrogen (NADPH and NADH) generated in seed embryos, and changed into red TF. The TF concentration was measured by light intensity at a wavelength of

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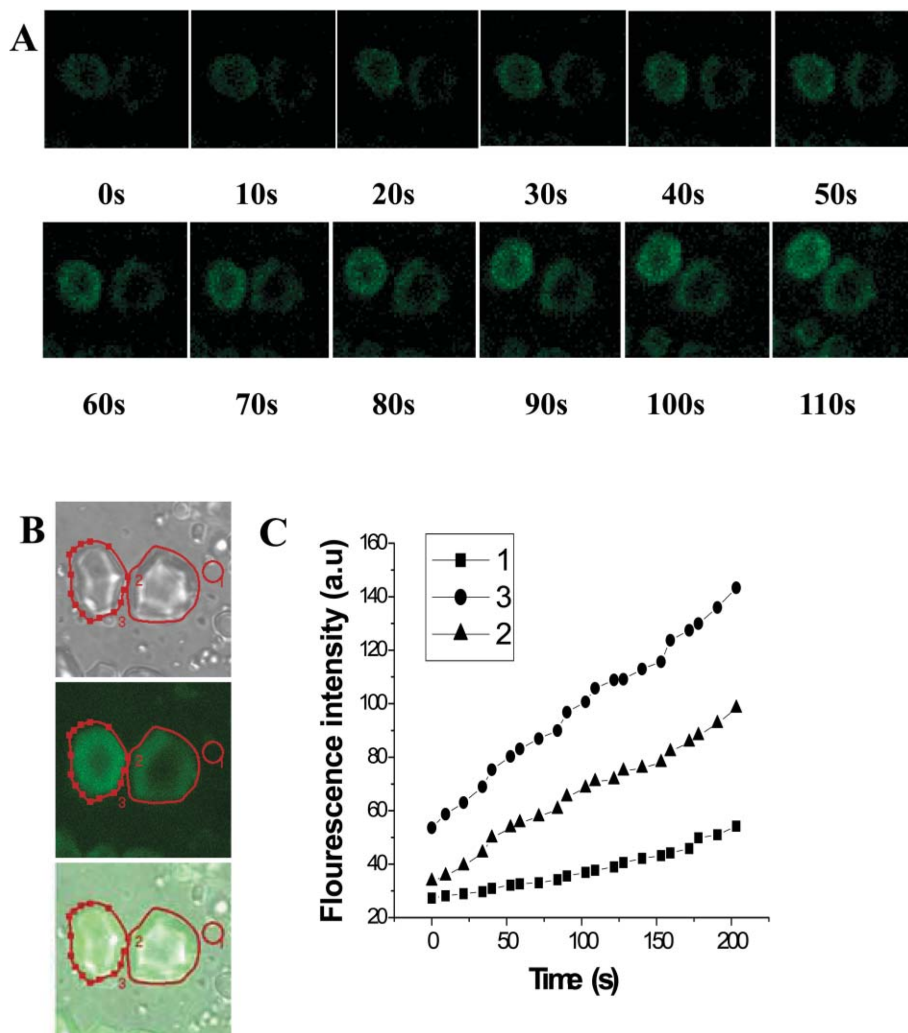

Fig. 4 Panels show differential interference contrast images of the same rice SEC. (A) Fluorescence images of DCF emission intensities. ROS formation in isolated rice seed embryo cells was observed under a LSM. H_2DCF-DA was added in PBS (pH 7.8). Excitation at 488 nm, collecting at the 500–550 nm bandpass. 40× (oil) object lens and the scan zoom is 10.7, time serial mode collects a picture every 10 s. (B) Corresponding to the time serial images of DCF staining in (A). (C) Dynamics of DCF fluorescence intensities corresponding to the images of DCF staining in (B).

485 nm. We used the value of OD_{485} to stand for the concentration of reductive hydrogen. We measured the concentration of reductive hydrogen of rice seeds harvested in different years in this way, and also examined MCLA-mediated CL induced by embryo cells. Interestingly, the same trend between MCLA-mediated CL and the concentration of reductive hydrogen was found (Fig. 6(A)). Correlative analysis indicated that this kind of MCLA-mediated CL had a positive correlation to reductive hydrogen concentration (Fig. 6(B)), correlation coefficient $R > 0.99$.

Discussion

There are two possible pathways of formation $O_2^{\bullet-}$ during rice seed germination. One is the electron leakage from the respiration chain of mitochondria to the dissociative oxygen, the other one is NADPH oxidase-catalyzed $O_2^{\bullet-}$-synthesis. Dry seeds exist generally in a glassy (or vitrified) state. The high viscosity of the glassy state would be expected to have a retarding effect on deteriorative reactions in the cytoplasm.[20] The amount and activity of mitochondria is very low in dry seeds. It is impossible that the superoxide anion during the immediate imbibition is produced by the respiration chain of mitochondrial electron leakage, another supporting reason is that the superoxide anion generated by mitochondrial electron leakage is difficultly released to the outside of the cell within the short lifetime of the superoxide anion.

Based on the fact that MCLA-mediated chemiluminescence, induced by superoxide anion generated from rice seed during early imbibition, could be dramatically abolished by SOD, a scavenger of superoxide anion, also by DPI, a specific inhibitor of NADPH oxidase (Fig. 1). This inhibitor and catalase, a scavenger of hydrogen peroxide, also suppressed ROS oxidizing H_2DCF-DA into DCF fluorescence (Fig. 2). We could conclude that in dry rice seeds existed intrinsic NADPH oxidase activity and this was the prominent source of superoxide anion. In the SPM reconstruct experiment, plasma membrane protein added by NDAPH could generate ROS and this course could be suppressed by NADPH oxidase inhibitors (Fig. 3). Taking the above data together, we could further conclude that the NADPH oxidase

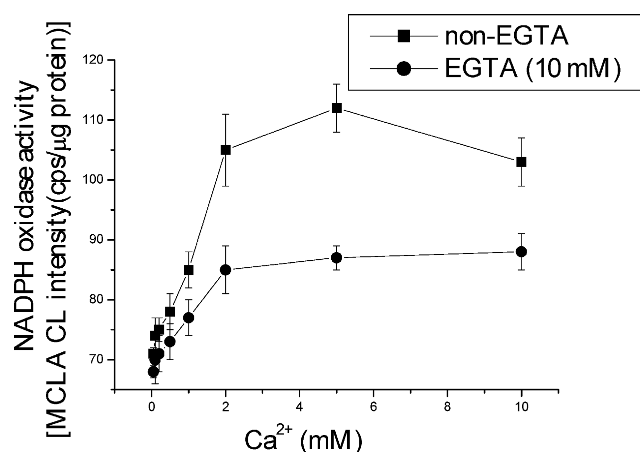

Fig. 5 NADPH oxidase activity in isolated rice seed membranes. NADPH oxidase activity was assayed in membranes of the upper phase of aqueous two-phase partitioned membranes. $O_2^{\bullet-}$ induced MCLA chemiluminescence is shown for increasing concentration of calcium ions. Where indicated 10 mM EGTA was added. Data are mean ±SE of four repeats and represent one of three different experiments that yielded similar results.

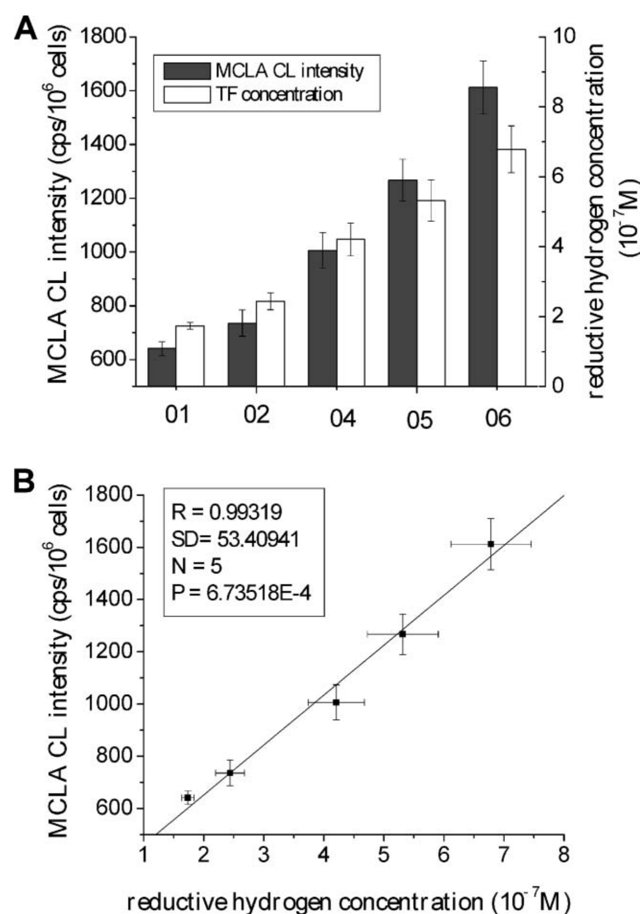

Fig. 6 (A) The reductive hydrogen concentration measured through the TF production from TTC reduced by seed embryo cells of different age, and MCLA-mediated CL induced by them. Bars 1, 2, 3, 4 and 5 stand for year of harvest 2001, 2002, 2004, 2005 and 2006, respectively. (B) Linear analysis of the relationship between seed embryo cells induced MCLA-mediated CL and the reductive hydrogen concentration.

activity intrinsically existed in rice seeds. Meanwhile, we observed under the LSM that the ROS located at the plasma membrane (Fig. 4). This provided further evidence that rice seeds contain intrinsic NADPH oxidase.

During the early period of imbibition, the increase of cytosolic calcium concentration $[Ca^{2+}]_{cyt}$ is mediated by Ca^{2+} released from internal stores.[21–24] The increase of $[Ca^{2+}]_{cyt}$ activates the activity of plasma membrane NADPH oxidase to catalyze NADPH and O_2 to generate ROS, the ROS activate plasma membrane Ca^{2+}-permeable (^{I}Ca) channels and then induce the Ca^{2+} influx. In this way a potential self-amplifying loop is formed which leads to the rapid formation of abundant ROS during early seed imbibition. Our experiment indicated that Ca^{2+} promoted the activity of NADPH oxidase (Fig. 5), so, there exists an intrinsic mechanism to generate ROS during early imbibition of seeds.

NADPH oxidase serves as a sensor for NADPH

The relationship between MCLA-mediated chemiluminescence and NADPH concentration can be expressed as follows:

$$\mathrm{NADPH} + \mathrm{O_2} \xrightarrow{\text{NADPH oxidase}} \mathrm{NADP^+} + \mathrm{H^+} + \mathrm{O_2^{\bullet-}} \quad (1)$$

$$\mathrm{MCLA} + \mathrm{O_2^{\bullet-}} \xrightarrow{k_1} \mathrm{MCLA^-} + \mathrm{O_2} + h\nu_{(\mathrm{CL})} \quad (2)$$

The equation for the kinetics of bisubstrate enzyme-catalyzed reaction (1) can be given by:

$$\frac{d[O_2^{\bullet-}]}{dt} = v = \frac{v_{max}[\mathrm{NADPH}][\mathrm{O_2}]}{K_m^A[\mathrm{O_2}] + K_m^B[\mathrm{NADPH}] + [\mathrm{NADPH}][\mathrm{O_2}]} \quad (3)$$

where v_{max} is the maximum rate or maximum velocity, $K_m{}^A$ is the Michaelis constant for NADPH, $K_m{}^B$ is the Michaelis constant for O_2.

Under the measurement conditions the intensity of oxygen, $[O_2]$, is invariable. The value of v_{max} stand for the activity of NADPH oxidase, which reaches at the culmination after maturation and decline during the storing. On the assumption that the activity of NADPH oxidase has not declined, we can consider eqn (1) as a function that the ratio of superoxide anion production $\frac{d[O_2^{\bullet-}]}{dt}$ changed with NADPH concentration [NADPH], the intensity of $O_2^{\bullet-}$ ($[O^{\bullet-}]$) fluctuates with [NADPH], the intensity of MCLA-mediated chemiluminescence is real-time reflects $[O^{\bullet-}]$. We can conclude there exists a positive correlation between [CL] and [NADPH]. If NADPH oxidase activity decreased during the storage, the decline of $O_2^{\bullet-}$ production would increased. We can conclude that there is a positive correlation between $O_2^{\bullet-}$-generation during early imbibition and seed vigor. NADPH oxidase catalyzes NADPH and O_2 to generate superoxide anions, so NADPH oxidase present in rice seeds serves as an intrinsic NADPH sensor. Therefore the production of superoxide anion is an indicator of the NADPH concentration in rice seeds, or of the combined effects of the change of NADPH oxidase and NADPH, which are directly related to seed vigor.

During the germination, the primary pathway of NADPH generation is pentose phosphate pathway, and glucose-6-phosphate dehydrogenase is the pivotal enzyme for NADPH generation. NADPH generated by this way provided enough reductive power for biosynthesis that is vital for the functions of the biomolecule

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during seed germination. NADPH is an index of seed vigor.[26] It has been reported that glucose-6-phosphate dehydrogenase activities were higher in seeds showing high germination capacity.[27] Namely, the NADPH concentration is higher in the high vigor seed. For NADPH, there exists a positive contribution to reductive hydrogen. It is difficult to detect NADPH directly, but we can measure the reductive hydrogen (NADPH and NADH) through the TTC reduction test. Even though it has not separated NADPH from NADH, there exists a decline trend for NADPH some as NADH in seeds with different degree of vigor.[26,27] So we can investigate the relationship between reductive hydrogen (NADPH and NADH) instead of NADPH and the superoxide anion induced MCLA-mediated CL in seed embryo cells harvested in different years (Fig. 6) to confirm the ratiocination that the production of superoxide anion can reflect the NADPH concentration.

Conclusions

Rice seeds contain intrinsic NADPH oxidase, and its activity is activated and amplified rapidly by certain pathways during early imbibition of seeds. The NADPH oxidase catalyzes NADPH to generate superoxide anion, and this superoxide anion can be released outside. The superoxide anion can reflect NADPH concentration, and it can be sensitively measured by MCLA-mediated chemiluminescence using a single photon counting apparatus.[1,25] Under these conditions, NADPH oxidase plays a role as an intrinsic NADPH sensor, so superoxide can serve as a rapid index of seed vigor. These results offer a theoretical foundation for the development of a rapid seed vigor test that monitors the superoxide anion.

Abbreviations

EGTA	Ethylene glycol bis(2-aminoethyl)tetraacetic acid
FDA	Fluorescence diacetate
Suc	Sucrose
TTC	2,3,5-Triphenyltetrazoliumchloride
TF	Triphenyl formazan
MCLA	2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo(1,2-α)pyrazin-3-one
CL	Chemiluminescence
H_2DCF-DA	Dichlorofluorescein diacetate
DCF	Dichlorofluorescein
ROS	Reactive oxygen species
DPI	Diphenylene iodonium
QA	Quinacrine
Tri	Tris(hydroxymethyl)aminomethane

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