Phosphatidylinositol 3-Kinase Plays a Vital Role in Regulation of Rice Seed Vigor via Altering NADPH Oxidase Activity

Jian Liu, Jun Zhou, Da Xing*

MOE Key Laboratory of Laser Life Science and Institute of Laser Life Science, College of Biophotonics, South China Normal University, Guangzhou, China

Abstract

Phosphatidylinositol 3-kinase (PI3K) has been reported to be important in normal plant growth and stress responses. In this study, it was verified that PI3K played a vital role in rice seed germination through regulating NADPH oxidase activity. Suppression of PI3K activity by inhibitors wortmannin or LY294002 could abate the reactive oxygen species (ROS) formation, which resulted in disturbance to the seed germination. And then, the signal cascades that PI3K promoted the ROS liberation was also evaluated. Diphenylene iodonium (DPI), an NADPH oxidase inhibitor, suppressed most of ROS generation in rice seed germination, which suggested that NADPH oxidase was the main source of ROS in this process. Pharmacological experiment and RT-PCR demonstrated that PI3K promoted the expression of Os rboh9. Moreover, functional analysis by native PAGE and the measurement of the 2, 3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazo-lium-5- carboxanilide (XTT) formazan concentration both showed that PI3K promoted the activity of NADPH oxidase. Furthermore, the western blot analysis of OsRac-1 demonstrated that the translocation of Rac-1 from cytoplasm to plasma membrane, which was known as a key factor in the assembly of NADPH oxidase, was suppressed by treatment with PI3K inhibitors, resulting in the decreased activity of NADPH oxidase. Taken together, these data favored the novel conclusion that PI3K regulated NADPH oxidase activity through modulating the recruitment of Rac-1 to plasma membrane and accelerated the process of rice seed germination.

Introduction

Seed germination is a complex event, which commence with the uptake of water by the quiescent dry seed and terminate with the elongation of the embryonic axis [1]. This process is influenced by various factors, including some signaling molecules, such as reactive oxygen species [2] and NO [3], several plant hormones, such as abscisic acid (ABA) [4], gibberellic acid (GA) [5] and ethylene [6], and other factors, for instance, HCN [7] and water channel proteins [8]. In addition, some lipids, for example, Phospholipase D (PLD) and its product phosphatidic acid (PA), are also indicated to be involved in seed germination [9]. PI3K as a key role in the regulation of lipid signal has been reported in plant physiology. However, there is no thorough study on the relationship between PI3K and seed germination.

In mammalian cells, there are three types of PI3K with different substrate specificities [10–12]. In plant cells, only type III PI3K, which phosphorylates the D-3 position of phosphoinositides, has been identified. The product of PI3K is Phosphatidylinositol 3-phosphate (PI3P), which is present at very low level in plant cell but turn over rapidly following the alteration of external environment [13–17]. PI3K and its product have been proved to be involved in various physiological events and stress responses [18], including root hair growth [19], root nodule formation [20], stomatal closing movement [21], nuclear transcription [12], auxin-induced reactive oxygen species production and root gravitropism [22], pollen development [23], actin filament reorganization [24], endocytosis of plasma membrane and the salt-stress-induced production of ROS [25]. However, in seed germination, there is no elaborate study about the function of PI3K. In particular, how PI3K and its downstream pathway involved in modulating seed germination remains unknown.

NADPH oxidase, which plays a significant role in generating ROS, has been implicated in seed germination [6,8,26]. In mammalian cells, PI3K can mediate the recruitment of the Rac-1 GTPase and the oxidase submits p47phox to the cell membrane, which affects the assembly of NADPH oxidase complex and eventually the activity of NADPH oxidase [27,28]. In addition, phosphoinositide products of PI3Ks can directly bind to the PX domains of the oxidase subunits p40phox and p47phox, which influences their abilities to translocate p67phox and finally alters the activity of NADPH oxidase [29,30]. In plant cells, one type of PI3K related to yeast Vps34 has been identified and seems to have more extensive functions [12]. Moreover, recent findings have proved that Rho GTPase, especially Rac1, is involved in the regulation of NADPH oxidase activity in rice [31,32]. However, there is still rare information about the association of PI3K with NADPH oxidase activity in plants.
In this study, we revealed that PI3K altered the quantity of ROS and played an essential role in rice seed germination, through the regulation of the expression and activity of NADPH oxidase. It was also characterized how PI3K regulated the activity of NADPH oxidase. Finally, we proposed a novel hypothetical model that PI3K regulated the translocation of Rac-1 from cytoplasm to plasma membrane, resulting in activated assembly of NADPH oxidase in rice seed germination.

Results

Changes of PI3K expression in rice seed after imbibition and the expression of PI3K is regulated by Ca\(^{2+}\)

To determine the dynamic expression of PI3K in rice seed germination, the total RNA was extracted from the rice seed embryo after imbibition. The result of RT-PCR analysis suggested that PI3K might involve in the regulation of seed germination. Meanwhile, it was found that the expression of PI3K was increased with the prolongation of imbibition time (Figure 1A). This expression peaked at 12 h and reached approximately 30 times as many as seeds without treatment (Figure 1C). Interestingly, low concentration of Ca\(^{2+}\) (below 10 mM) could further increase the expression of PI3K in rice seeds after germinated for 12 h. However, when the concentration of Ca\(^{2+}\) was up to 20 mM, the PI3K expression began to decrease slightly compared with the treatment with 10 mM CaCl\(_2\) (Figure 1B, D).

LY294002 and Wortmannin inhibit rice seed germination

Next, Wortmannin and LY294002, two kinds of PI3K inhibitors with different action mechanisms, were used to test the role of PI3K in the de-coated (without pericarp) rice seed germination [21]. Following treatment with 60 µM LY294002 at 27°C for four days, the seed germination rate decreased to about 67.3% compared with the control. And it was further decreased to 54.0% when the concentration of LY294002 was increased to 90 µM (Figure 2A). The seed germination rate was 55.7% of the control in the presence of 20 µM Wortmannin and it would be decreased to 31.7% of the control with 30 µM Wortmannin (Figure 2B). Dynamics of seed germination revealed that, relative to the control, 60 µM LY294002 or 20 µM Wortmannin displayed both significant inhibitory effects on the seed germination at any time point (Figure 2C). From these results, we reasonably concluded that PI3K played a positive role in rice seed germination.

LY294002 and Wortmannin inhibit ROS production

PI3K inhibitors suppressed the ROS production in guard cell, root hair, and pollen tube [18,19,23]. However, it was uncertain that PI3K inhibitors restrained the ROS formation in rice seed germination. In our case, it was found that exogenous hydrogen peroxide (H\(_2\)O\(_2\)) could partly rescue the inhibitory effects of PI3K inhibitors on rice seed germination (Figure S1A). This finding allowed us to assume the possible association of PI3K with ROS production during rice seed germination.

Figure 1. Changes of PI3K expression in rice seed after imbibition. RT-PCR assayed the expression of PI3K from rice seed, which was imbibed from 0 hour to 12 hours in the presence (A) or absence (C) of 20 mM CaCl\(_2\). UBQ, mRNA of rice ubiquitin was used as control. (C) Different concentration of CaCl\(_2\) was used to treat with seed in 12 hours imbibition, then isolated the embryo and analyzed the alteration of PI3K expression by RT-PCR. (B, D) Quantitive analysis of the result of RT-PCR in statistical method. Data are means of three replicates ± SD. * indicates the values that are significantly different from control (P<0.05).

doi:10.1371/journal.pone.0033817.g001

NADPH Oxidase Regulated by PI3K Promote Seed Vigor
To further investigate this relationship between PI3K and ROS, ROS probe H$_2$DCFDA was used to examine the characteristics of ROS production under the treatment of pharmacological inhibitors of PI3K LY294002 (60 μM) or Wortmannin (20 μM). Results demonstrated that, during the imbibition period of 48 h, treatment with PI3K inhibitors resulted in lower level of ROS production compared with the control (Figure 3A). Meanwhile, the change of superoxide anion was also examined through NBT staining [33] and XTT test [34]. As shown in Figure 3B, 3C, the alteration of superoxide anion was similar with the change of ROS level. Interestingly, PI3K inhibitors seemed to suppress ROS not only by NADPH oxidase but also other sources (Figure S2). The above findings led us to get the conclusion that PI3K inhibitors could suppress the formation of ROS in rice seed germination.

NADPH oxidase is an important source of ROS in rice seed germination

Several studies have showed that ROS plays a key role in seed germination [7,35,36]. Here, we also found that rice seed germination could be inhibited by ROS scavengers, 10 mM KI or 1 Mm ASA (Figure S1B). Subsequently, 100 μM diphenylene iodonium (DPI), one of the highly effective inhibitor of NADPH oxidase [27], was used to explore whether NADPH oxidase is involved in ROS production responsible for rice germination. As shown in Figure S1B, the germination rate in rice seed treated with DPI was lower than that of control. Interestingly, exogenous H$_2$O$_2$ (20 mM) could partly rescue the decrease in seed germination caused not only by KI and ASA but also by DPI (Figure S1B). These phenomena indicated that NADPH oxidase might be the important source of ROS formation in rice seed embryo under germination.

To prove this hypothesis, the concentration of superoxide anion was under determination in the absence and presence of 60 μM LY294002 or 20 μM Wortmannin. As shown in Figure 4A, NBT was applied to evaluate the quantity of superoxide anion. DPI (100 μM) was added before NBT staining. Compared with control, exogenous DPI could significantly suppress the formation deposits in the embryos.

To further verify this phenomenon, superoxide anion was quantified by XTT [34]. As was illustrated in Figure 4B, under the treatment of PI3K inhibitors LY294002 (60 μM) or Wortmannin (20 μM), the formation of superoxide anion was obviously restricted in contrast with control. These observations confirm the idea that NADPH oxidase is an important source of ROS in seed germination.

LY294002 and Wortmannin inhibit the expression of NADPH oxidase

The earlier studies have reported that NADPH oxidase-generated ROS plays a key role in seed germination [7,35,36]. As mentioned above, PI3K inhibitors can prevent ROS production during rice seed germination. Therefore, it is worth assuming that PI3K might control ROS level via regulating NADPH oxidase. In the previous study, it has been proved that PI3K is associated with nuclear transcription sites in higher plant [12]. Thus, we examined whether PI3K regulated the transcription of NADPH oxidase in rice seed germination. Firstly, in order to investigate the types of NADPH oxidase in the rice seed germination, the RNA was extracted from wild type rice seed imbibed for 5 days after imbibition. Means of three replicates ± SD. * indicates the values that are significantly different from control (P<0.05).

doi:10.1371/journal.pone.0033817.g002
assayed during the initial 24 h of rice seed germination (Figure 5C, D). To investigate whether PI3K promote the transcription of NADPH oxidase in the rice seed germination, the expression of NADPH oxidase was examined in the presence of 60 μM LY294002 or 20 μM Wortmannin. As shown in Figure 5E, F, the PI3K inhibitors notably restrained the expression of NADPH oxidase Osrboh9 and slightly suppressed the expression of Osrboh4 in comparison with other types of NADPH oxidase. On the basis of the above results, we drew the conclusion that PI3K could promote the transcription of Osrboh4 and Osrboh9 other than the rest types of NADPH oxidase.

**PI3K inhibitors suppress the activity of NADPH oxidase**

The effect of PI3K on the activity of NADPH oxidase was also examined. At first, in situ gel NBT was performed with the isolated plasma membrane, which was obtained from the rice seed embryo imbibed for 24 h in the absence and presence of 60 μM LY294002 or 20 μM Wortmannin. As shown in Figure 6A, the band from the control PM was obviously stronger than that from the PM in the presence of 60 μM LY294002 or 20 μM
The NADPH oxidase enzymatic activity was also examined by the measurement of the XTT formazan concentration with 60 μM LY294002 or 20 μM Wortmannin. The experimental result showed that the LY294002 and Wortmannin inhibited NADPH oxidase activity by 36.6% and 18.2%, respectively (Figure 6B). It appears therefore, that PI3K promoted the activity of NADPH oxidase in rice seed germination.

PI3K is required for subcellular translacaiton of Rac-1

In mammalian cells, it has been proved that PI3K class I can regulate the translocation of cytosolic factors, such as Rac-1. In fact, it is necessary for the assembly of the active NADPH oxidase complex to translocate Rac-1 to the cell membrane in rice cells [31]. However, it has been unclear whether PI3K regulates NADPH oxidase activity through mediating the translocation of Rac-1.
To investigate the possible mechanisms of Rac1 regulated by PI3K, western blot was used. The experiment was performed with total or membrane protein isolated from rice seed embryo cells imbibed for 24 h with or without 60 μM LY294002 or 20 μM Wortmannin. Western blot analysis of membrane protein indicated that the translocation of Rac-1, following the treatment of LY294002, was suppressed compared with control, whereas the quantity of Rac-1 from the total protein was not obviously altered. In addition, compared with that of LY294002, another PI3K inhibitor Wortmannin seemed to abate the amount of total Rac1 while it reduced the amount of membrane Rac1 severely (Figure 7A, 7B). These results (Figure 7C, 7D) confirmed the speculation that PI3K promoted the translocation of Rac-1 to the membrane and thus facilitated the activity of NADPH oxidase.

Discussion

Although many papers reported that the seed of PI3K mutant showed a reduced germination rate compared with wild type [23], it has rarely been directly assayed so far. In this study, we elaborately characterized the effects of PI3K on the rice seed germination by promoting NADPH oxidase activity. LY294002 and Wortmannin, two sorts of PI3K specific inhibitor, obviously inhibited the rice seed germination. Our mainly study here focused on the inhibition of ROS generation (Figure 3) and the relationship between PI3K and NADPH oxidase in seed germination (Figure 6, 7).

PI3K activity is closely correlated with rice seed germination

In plant cells, the level of PI3P, as the product of PI3K, were relatively low, but turn over rapidly following the alteration of external environment [14]. This character determined that the level of PI3P maybe has more sensitive response to the external stimulation. Thus, we firstly examined the expression of PI3K in rice seed germination (Figure 1A). From 0 hour to 12 hours, the expression of PI3K almost raised thirty times than initial expression, which obviously indicated the key role of PI3K in rice seed germination. Interestingly, the exogenous Ca2+ promoted the expression of PI3K (Figure 1B). As we know, the level of Ca2+ rapidly increased following by the uptake of water in the early germination. Here, PI3K seemed to be one of the important relay station of Ca2+ signal. In previous publication, it had been reported that Wortmannin and LY294002 inhibited Ca2+ oscillation induced by ABA [37], which supported the role of PI3P in ABA-induced Ca2+ oscillation. In contrast with the above studies, our results showed that Ca2+ promoted the expression of PI3K. Meanwhile, it was reported that PI3K could also raise the levels of intracellular Ca2+ [37]. Therefore, we speculated that PI3K and Ca2+ seemed to have a feedback regulated relationship. This cycle rapidly altered the total quantities of PI3K and Ca2+, which laid the foundation for fulfilling their functions in the seed germination.

To provide direct evidence for the role of PI3K on the rice seed germination, Wortmannin and LY294002, two kinds of PI3K inhibitors with different action mechanisms, were used to treat with rice seed (Figure 2A, B). 60 μM LY294002 inhibited the seed germination by 67.3%, while 20 μM Wortmannin suppressed the seed germination by 55.6%. In addition, this effect of PI3K inhibitors on rice seed germination was concentration dependent. To investigate the dynamic alteration of rice seed germination following by the prolongation of imbibition under the treatment with PI3K inhibitors, the germination rate from 1 day to 5 days

---

Figure 6. PI3K inhibitors suppress the activity of NADPH oxidase in seed germination. (A) In situ gel NBT assay. The PM fraction was separated by native PAGE, and the gel was incubated with NBT solution, then with 0.2 mM NADPH until the appearance of blue formazan bands was observed. The reaction was stopped by immersion of the gel in distilled water. (B) Quantitative analysis of the result of native PAGE in statistical method. (C) The seed embryos was immersed in the solution within the PI3K inhibitor for 12 hours, then isolate the plasma membrane, Plasma membrane vesicles (4 mg protein) were incubated with XTT at 25°C for 10 min. The reaction solution was used for spectrophotometric analysis of XTT formazan absorbance at A470. NADPH oxidase activity was expressed as ΔA470 per mg protein per min (ΔA470 represents the difference of XTT formazan absorbance at 470 nm in the presence and absence of superoxide dismutase [SOD]). Means of three replicates ± SD and the pictures represent typical examples. * indicates the values that are significantly different from control (P<0.05).

doi:10.1371/journal.pone.0033817.g006
showed that PI3K inhibitors suppressed the rice seed germination during these processes.

**PI3K-Rbohs interaction suggested a novel regulatory mechanism for rice seed NADPH oxidase**

Many articles have reported that ROS played an important role in seed germination. In these processes, ROS could interplay with the hormonal signaling pathway, such as abscisic acid (ABA), gibberellins (GAs) and ethylene [35,38]. The accumulation of ROS could be beneficial for seed germination through controlling the cell redox status. Various transcription factors had been shown to sense ROS via the formation of disulfides involving thioredoxin and glutaredoxin [7,39]. In addition, the established mechanisms of ROS transduction pathway, which involved MAP kinase cascade activation, inhibition of phosphatases, activation of Ca^{2+} channels and Ca^{2+} binding proteins, also had an important function in seed germination.

In previous studies, it had been reported that PI3K could regulate the formation of ROS in majority of plant tissues, such as root hair [19], pollen tube [23] and guard cell [21]. Our experiments proved that the exogenous H_2O_2 abated the inhibition of PI3K inhibitors during the rice seed germination (Figure S1A), which implied that ROS played a significant role by the regulation of PI3K. Thus we investigated whether PI3K promoted the generation of ROS in rice seed germination.

H_2DCFDA was used to determine the quantity of ROS. In contrast with the treatment of control, the ROS quantity of treatment was notably decreased. It seemed that PI3K inhibitors inhibited ROS formation. To further verify this point, superoxide anion was examined by NBT dyeing and the determination of XTT reduction product absorbance. Similar observation was obtained. Meanwhile, NADPH oxidase was also proved to be the important source of ROS in rice seed germination when used DPI, an NADPH oxidase inhibitor. On the basis of these results, the clearly signal pathways between PI3K and NADPH oxidase was put on the agenda.

In the former publication, it had been shown that PI3K activity was associated with nuclear transcription [12]. Thus, we firstly investigated whether PI3K regulated the transcription of NADPH oxidase. From the analysis of the rice genome database, nine Os rboh genes were identified. RT-PCR analysis showed that only rboh2, rboh4, rboh5 and rboh9 were expressed in rice seed germination. The determination of the NADPH oxidase expression under the treatment with PI3K inhibitors verified that PI3K inhibitors inhibited the expression of rboh4 and rboh9. It seemed that PI3K could affect the formation of ROS by regulating rboh4 and rboh9 expression.

At the same time, the influence of PI3K inhibitors on the activity of NADPH oxidase was also performed. Two approaches, native PAGE and the determination of XTT reduction product...
absorbance, both demonstrated that PI3K inhibitors obviously suppressed the activity of NADPH oxidase.

However, how PI3K regulates the activity of NADPH oxidases is still unknown. In human myeloid cell, PI3 P was associated with the noncatalytic component p40phox of the NADPH oxidase, and then affected the translocation of p67phox to p47phox, finally altered the activity of NADPH oxidase [29]. Moreover, in phagocytic cell, PI3K could influence that plasma membrane recruited Rac-1 and p40phox [27], which were the necessary components of NADPH oxidase. The homolog of p40phox has not been reported in plant, but Rac-1 had been proved as an important role on the activity of NADPH oxidase in rice. Therefore, it was necessary to investigate on the relationship between PI3K and Rac-1.

The analysis of western-blot was shown by using Rac-1 antibody. As expected, under control conditions, the percentage of Rac-1, which was transmitted to membrane, was more than that of inhibitor treatment. The translocation of Rac-1 to the membrane was suppressed, but total Rac-1 in the homogenate was not changed obviously under the treatment of LY294002. In addition, compared with that of LY294002, another PI3K inhibitor Wortmannin seemed to alter the amount of total Rac-1 while it reduced the amount of membrane Rac-1 severely (Figure 7). The difference in the drug used might be result in the difference. As we know, LY294002 is a special inhibitor of PI3K, but Wortmannin is a fungal toxin that inhibits not only PI3K but also PI4K and PIPK [19]. Based on these results, it was proved that PI3K promoted the activity of NADPH oxidase through mediating the translocation of Rac-1 to membrane. However, it would lead to a new question how PI3K regulate the translocation of Rac-1. In neutrophils, P-Rex, the member of Rho family GEFs, was strongly and directly activated by PtdIns(3,4,5)P3. And P-Rex also was the PI3K effectors, which led to the accumulation of oxidase. The homolog of p40phox has not been reported in plant, but Rac-1 had been proved as an important role on the activity of NADPH oxidase in rice. Therefore, it was necessary to investigate on the relationship between PI3K and Rac-1.

To test the effect of PI3K inhibitors on seed germination, LY294002 and Wortmannin at different concentration were used to incubate rice seeds at 28°C. In order to verify the importance of ROS on seed germination, two ROS scavenger ascorbic acid (ASA) (100 mM) and KI (10 mM) were used to treat the seeds. In addition, DPI (100 μM) was also used to test if NADPH oxidase was one of the major ROS sources. The average germination rate of the triplicate of 100 seeds was calculated every 24 h over a period of 5 days.

Materials and Methods

Plant material and chemicals

Rice (Oryza sativa L. Nipponbare) seeds were kindly provided by Professor L.X. Zeng (Guangdong Academy of Agricultural Sciences, China). H2DCFDA was obtained from Molecular Probes (Eugene, OR, USA). DPI, nitroblue tetrazolium (NBT) and XTT were purchased from Sigma-Aldrich (Shanghai, China). LY294002 and Wortmannin were obtained from BiYunTian (Nantong, China). Rac-1 antibody was bought from Cell Signalling Technology (USA).

Determination of superoxide anion and ROS in rice seed embryos

ROS release from embryos was determined by the fluorescence of DCF, which was the product of oxidation of dichlorofluorescin.
diacetate (H$_2$DCFDA), as described previously [43–45]. Briefly, the isolated embryos were cut longitudinally and incubated in 20 mM potassium phosphate buffer (pH 6.0) with H$_2$DCFDA at a final concentration of 5 μM for 20 min at 30°C on a shaker. And then 200 μl of the solution was taken and its fluorescence (excitation: 488 nm, emission: 525 nm) was measured within a few minutes using a microtiter plate reader (Tecan, microplate reader, infinite M200; Austria) [46].

To determine superoxide anion, after imbibitions with or without some inhibitors, batches of 100 embryos were incubated in 1 ml of K-phosphate buffer (20 mM, pH 6.0) containing 500 μM XTT in darkness at 25°C on a shaker for 3 h, and XTT reduction was determined at 470 nm. Blanks without plant material were run in parallel and used for subtracting spontaneous fluorescence changes [43]. The accumulation of superoxide anion was also monitored in situ by NBT as described previously [6,47]. Simply, seeds without capsules imbibed with DPI (100 μM) or LY294002 (60 μM) or Wortmannin (20 μM), and embryos were cut longitudinally and incubated with 6 mM NBT in 10 mM Tris-HCl buffer (pH 7.4) at 30°C for 2 h. Superoxide anion was visualized as deposits of dark-blue insoluble formazan compounds. Microscopic images were taken using a Zeiss microscope as described previously [48].

**Plasma membrane protein extraction from embryos**

Plasma membranes were isolated by an aqueous two-phase partitioning system according to the method reported previously with minor modifications [8,49]. The embryos were frozen in liquid nitrogen and fragmented using a pestle and mortar in a buffer (1.5 ml g$^{-1}$ fresh weight) containing 0.25 M sorbitol, 1 mM EGTA, 20 μM phenylmethylsulfonyl fluoride, 1% (w/v) PVP, 2 mM dithiothreitol, and 50 mM Tris-acetate, pH 7.5, and filtered through four layers of gauze. The resulting filtrate was centrifuged at 15,000 × g for 20 min. The recovered supernatant was centrifuged at 100,000 × g for a further 30 min and the resulting membrane pellet (microsomal membranes) was gently resuspended in 5 mM potassium phosphate buffer (pH 7.8) including 330 mM Suc, 5 mM KCl, 1 mM DTT, 0.1 mM EDTA and protease inhibitors. The suspension was then fractionated by the aqueous two-phase partitioning method according to the batch procedure [50]. Plasma membrane vesicles were prepared using a 10 g aqueous two-phase partitioning system. Resuspended microsomal fractions were mixed with 6.5% (w/w) PEG 3350, 6.5% (w/w) Dextran T-500, 5 mM potassium phosphate buffer (pH 7.8) including 330 mM Suc, 5 mM KCl, 1 mM DTT, and 0.1 mM EDTA and protease inhibitors. After mixing, the phases were separated by centrifuging at 4,000 × g for 5 min. The upper phase, enriched in plasma membrane (PM) vesicles, was repartitioned twice with fresh lower phase without PEG and, after dilution with 0.25 M sorbitol, 1 mM EGTA, 2 mM MgCl$_2$, 2 mM dithiothreitol, and 20 mM Tris-acetate, pH 7.5, and then the PM vesicles were collected by centrifuging at 100,000 × g for 45 min. The pellets were then resuspended in Tris-HCl dilution buffer and stored at −80°C for further analysis. All procedures were carried out at 4°C.

**Determination of PM NADPH oxidase activity**

The NOX activity of PM vesicles was determined by an assay based on reduction XTT by superoxide anion [34]. The analysis reaction medium contained 10 μg upper phase proteins, 0.3 mM XTT, and 0.18 mM NADPH in 1 ml 50 mM Tris-HCl buffer (pH 7.4) with 100 μM CaCl$_2$ or 10 mM EGTA. Protein concentrations were determined by the approach of Bradford [51]. XTT reduction was determined at 470 nm in the presence and absence of 50 units SOD.

**In situ gel NBT assay**

The NADPH-dependent superoxide anion coming from the isolated membrane fractions were assayed in native gels by a modified NBT reduction method [34,52]. Protein samples from each fraction (20 μl per lane) were separated in a 7.5% (w/v) polyacrylamide gel.
separating gel and 4% (w/v) stacking gels with or without 0.1% (v/v) CHAPS at 4°C. The gel was incubated in the dark with 0.2 mM NBT solution (50 mM Tris–HCl, 0.2 mM NBT, 0.1 mM MgCl₂, and 1 mM CaCl₂, pH 7.4) for 20 min, and then with 0.5 mM NADPH until the appearance of blue formazan bands was observed. The reaction was stopped by immersion of the gel in distilled water.

Western blot and coomassie staining of proteins
To obtain the optimal anti-body, amino acid sequences of Rac-1 in different species was compared using DNAMan (Figure S3). Membrane proteins and total proteins were separated by 12% SDS-PAGE and transferred onto PVDF membranes (American Pharmacia Biotech) and subjected to immunodetection with Rac-1/Cdc42 polyclonal antibody, which was the result of Rac-1 sequence comparison from different species. The Rac-1/Cdc42 antibody could recognize endogenous levels of total rac-1 and cdc42 protein. The antigen-antibody complex was visualized with anti-rabbit secondary antibody and enhanced chemiluminescence. The Coomassie Brilliant blue-stained gel was used to show that an equal amount of proteins [53]. All the experiments took three independent repetitions.

Supporting Information
Figure S1 H₂O₂ abate the inhibition of PI3K inhibitor on the rice seed germination. Germination of rice seed embryos came from the same seed lot at 27°C in the dark. Rice seed treated with 10 mM KI, 1 mM ASA, 100 μM DPI, 20 μM Wortmannin, 60 μM LY294002, KI and H₂O₂ combined (10 μM KI+10 mM H₂O₂), ASA and H₂O₂ combined (1 mM ASA+10 mM H₂O₂), DPI and H₂O₂ combined (100 μM KI+10 mM H₂O₂), Wortmannin and H₂O₂ combined (20 μM Wortmannin+10 mM H₂O₂), LY294002 and H₂O₂ combined (60 μM LY294002+10 mM H₂O₂), respectively. The germination of rice seed after 3 days imbibition was counted. Data are means of three replicates ± SD. * indicates the values that are significantly different from control (P<0.05).

References
1. Bewley JD (1994) Seeds germination and dormancy. Plant Cell 9: 1055-1066.
2. Bailly C, El-Maarouf-Bouteau H, Corbineau F (2008) From intracellular signaling networks to cell death: the dual role of reactive oxygen species in seed physiology. C R Biol 331: 806–814.
3. Sarah G, Hou GC, Baird LM, Mitchell RB (2007) ABA, ROS and NO are key players during switchgrass seed germination. Plant Signal Behav 2: 492–493.
4. Goggin DE, Steadman KJ, Emery RJN, Farrow SC, Benech-Arnold RL, et al. (2009) The mechanisms involved in seed dormancy alleviation by hydrogen peroxide. J Exp Bot 61: 2979–2990.
5. Liu YG, Ye NH, Liu R, Chen MX, Zhang JH (2010) H₂O₂ mediates the regulation of ABA catalysis and GA biosynthesis in Arabidopsis seed dormancy and germination. J Exp Bot 61: 2979–2990.
6. Oracz K, El-Maarouf-Bouteau H, Farrant JM, Cooper K, Belghazi M, et al. (2007) ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation. Plant Cell 19: 452–465.
7. Oracz K, El-Maarouf-Bouteau H, Kramer I, Bogatek R, Corbineau F, et al. (2009) The mechanisms involved in seed dormancy alleviation by hydrogen cyanide unravel the role of reactive oxygen species as key factors of cellular signaling during germination. Plant Physiol 150: 494–505.
8. Liu HY, Xin Y, Cui DY, Sun MH, Sun WN, et al. (2007) The role of water channel proteins and nitric oxide signaling in rice seed germination. Cell Res 17: 638-649.
9. Katagiri T, Takahashi S, Shinozaki K (2001) Involvement of a novel Arabidopsis phosphophosphate D, AtPLDDelta, in dehydration-inducible accumulation of phosphatidic acid in stress signaling. Plant J 26: 595-605.
10. Toker A, Cantley LC (1997) Signaling through the lipid products of phosphoinositide-3-OH kinase. Nature 387: 673-676.
11. Backer JM (2000) The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochim J 340: 1-11.
12. Bunney TD, Watkins PA, Beven AF, Shaw PJ, Hernandez LE, et al. (2000) Association of phosphatidylinositol 3-kinase with nuclear transcription sites in higher plants. Plant cell 12: 1679-1688.

Figure S2 Treatment with PI3K inhibitors decreases intracellular ROS level based on the treatment of DPI. Rice seed embryos were pretreated with 100 μM DPI, DPI and Wortmannin combined (100 μM DPI+20 μM Wortmannin), DPI and LY294002 combined (100 μM DPI+60 μM LY294002), respectively. ROS was determined by H₂DCFDA. DCF fluorescence was measured using a microtiter plate reader as described in Materials and Methods. Data are means of three replicates ± SD.

Figure S3 Comparison of amino acid sequences of Rac-1 in different species. To obtain the optimal anti-body, amino acid was compared using DNAMan, and the protein sequences used correspond to the following GenBank ID (from top to bottom): AAA36537, CAA40545, AAC49851, CAD24726, AAA96980, BAA84492.

Table S1 List of Primers used in semi-quantitative reverse transcription -polymerase chain reaction (RT-PCR). rboh gene was searched from the rice genome database and appraised nine genes. Compared with rboh34 gene in Arabidopsis, rboh gene was found from rice genome database. rboh gene was used as a control as described in the previous study.

Acknowledgments
We thank Prof. L.X. Zeng for providing rice seeds and appreciate the conversation with the members of our group in developing some of the ideas presented in this study, particularly the guidance of Dr. L.R. Zhang on this paper.

Author Contributions
Conceived and designed the experiments: JL, DX. Performed the experiments: JL, JZ. Analyzed the data: JL, JZ, DX. Contributed reagents/materials/analysis tools: JL, DX. Wrote the paper: JL, JZ, DX.

Author Contributions
Conceived and designed the experiments: JL, DX. Performed the experiments: JL, JZ. Analyzed the data: JL, JZ, DX. Contributed reagents/materials/analysis tools: JL, DX. Wrote the paper: JL, JZ, DX.
24. Choi Y, Lee Y, Jeon BW, Staiger CJ, Lee Y (2008) Phosphatidylinositol 3- and 4-phosphate modulate actin filament reorganization in guard cells of day flower. Plant Cell and Environ 31: 366–377.

25. Leshem Y, Seri L, Levine A (2007) Induction of phosphatidylinositol 3-kinase-mediated endocytosis by salt stress leads to intracellular production of reactive oxygen species and salt tolerance. Plant J 31: 185–197.

26. Sagi M, Fluhr R (2006) Production of reactive oxygen species by plant NADPH oxidases. Plant Physiol 141: 336–340.

27. Baumber AT, Freyhaus HT, Sauer H, Wartenberg M, Kappert K, et al. (2008) Phosphatidylinositol 3-kinase-dependent membrane recruitment of Rac-1 and p47phox is critical for alpha-platelet-derived growth factor receptor-induced production of reactive oxygen species. J Biol Chem 283: 7864–7870.

28. Baumber MA, Marimur R, Rosenthal KL, Sondek J, Lemmon MA, et al. (2003) Loss of phosphatidylinositol 3-phosphate binding by the C-terminal Tiam-1 pleckstrin homology domain prevents in vivo Rac-1 activation without affecting membrane targeting. J Biol Chem 278: 11457–11464.

29. Bissonnette SA, Glazier CM, Stewart MQ, Brown GE, Ellson CD, et al. (2008) Phosphatidylinositol 3-phosphate-dependent and -independent functions of p40phox in activation of the neutrophil NADPH oxidase. J Biol Chem 283: 2108–2119.

30. Ellson CD, Gobert-Gosse S, Anderson KE, Davidson K, Erdjument-Bromage H, et al. (2001) PtdIns(3)P regulates the neutrophil oxidase complex by binding to the PX domain of p40phox. Nat Cell Biol 3: 679–682.

31. Wang HL, Fraenkel R, Hayashi K, Tabata R, Yano T, et al. (2007) Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant cell 19: 4022–4034.

32. Jones MA, Raymond MJ, Yang ZB, Smirnoff N (2007) NADPH oxidase-dependent reactive oxygen species formation required for root hair growth depends on ROP GTase to its N-terminal extension. Plant cell 19: 4022–4034.

33. Hu LF, Liang WQ, Yin CS, Cui X, Zong J, et al. (2011) Rice MADS3 regulates ROS homeostasis during late anther development. Plant Cell 23: 515–533.

34. Sagi M, Fluhr R (2001) Superoxide production by plant homologues of the gp91phox NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiol 126: 1281–1290.

35. El-Maarouf-Bouteau H, Bailly C (2008) Oxidative signaling in seed germination and dormancy. Plant Cell Physiol 49: 1320–1329.

36. Liu XJ, Xing D, Li LL, Zhang LR (2007) Rapid determination of seed vigor and mitochondrial dysfunction that precede photosynthetic dysfunction and subsequent cell death. Plant Cell Physiol 49: 1092–1111.

37. Zhang LR, Xing D (2008) Methyl jasmonate induces production of reactive oxygen species and alterations in mitochondrial dynamics that precede photodynamic dysfunction and subsequent cell death. Plant Physiol 148: 1302–1312.

38. Potocky M, Jones MA, Bezvoda R, Smirnoff N, Zársky V (2007) Reactive Oxygen Species as Common Signals. Plant cell Physiol 44: 1092–1111.

39. Beyer WF, Jr., Fridovich I (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Anal Biochem 161: 559–566.

40. Potocky M, Jones MA, Bezvoda R, Smirnoff N, Zársky V (2007) Reactive oxygen species produced by NADPH oxidase are involved in pollen tube growth. New Phytol 174: 742–751.

41. Ohshima Y, Iwasaki I, Suga S, Murakami M, Inoue K, et al. (2001) Low aquaporin content and low osmotic water permeability of the plasma and vacuolar membranes of a CAM plant Graptopetalum paraguayense: comparison with radish. Plant Cell Physiol 42: 1119–1129.

42. Wilström P, Flygare S, Grøndal A, Larsson PO (1987) Magnetic aequorin two-phase separation: A new technique to increase rate of phase-separation, using destran-ferrofluid or lager iron oxide particles. Anal Biochem 167: 331–339.

43. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254.

44. Zheng Z, Ma C, Gao C, Li F, Qin J, et al. (2011) Efficient Conversion of Phenylpyruvic Acid to Phenyllactic Acid by Using Whole Cells of Bacillus coagulans SDM. PLoS ONE 6(4): e19030. doi:10.1371/journal.pone.0019030.

45. Li JS, Wang XM, Zhang YL, Jia HL, Bi YR (2011) cGMP regulates hydrogen peroxide accumulation in calcium-dependent salt resistance pathway in Arabidopsis thaliana roots. PloS 234: 709–722.