Nitric Oxide and Protein S-nitrosylation are Integral to Hydrogen Peroxide Induced Leaf Cell Death in Rice

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Appropriate journal categories:
System Biology, Molecular Biology and Gene Regulation
Environmental Stress and Adaptation

Running Title: S-nitrosylation is Involved in H₂O₂-Induced Leaf Cell Death in Rice
Nitric Oxide and Protein S-nitrosylation are Integral to Hydrogen Peroxide Induced Leaf Cell Death in Rice

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ABSTRACT

Nitric oxide (NO) is a key redox-active, small molecule involved in various aspects of plant growth and development. Here, we report the identification of an NO accumulation mutant noe1 (nitric oxide excess 1) in rice, the isolation of the corresponding gene and the analysis of its role in NO-mediated leaf cell death. Map-based cloning revealed that NOE1 encoded a rice catalase OsCATC. Further, noe1 resulted in an increase of hydrogen peroxide (H$_2$O$_2$) in the leaves, which consequently promoted NO production via activation of nitrate reductase (NR). Removal of excess NO reduced cell death in both leaves and suspension cultures derived from noe1 plants, implicating NO as an important endogenous mediator of H$_2$O$_2$-induced leaf cell death. Reduction of intracellular SNO (S-nitrosothiol) levels, generated by over-expression of OsGSNOR, which regulates global levels of protein S-nitrosylation, alleviated leaf cell death in noe1 plants. Thus, S-nitrosylation was also involved in light-dependent leaf cell death in noe1. Utilizing the biotin-switch assay, nanoliquid chromatography, and tandem mass spectrometry (LC/MS/MS), S-nitrosylated proteins were identified in both wild type and noe1 plants. NO targets identified only in noe1 plants included glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and thioredoxin (TRX), which have been reported to be involved in S-nitrosylation regulated cell death in animals. Collectively, our data suggest that both NO and SNOs are important mediators in the process of H$_2$O$_2$-induced leaf cell death in rice.
INTRODUCTION

Nitric oxide (NO) is a redox-active molecule that plays a key role in a broad spectrum of physiological and developmental functions throughout the plant life cycle. Thus, NO is thought to contribute to the control of germination, leaf expansion, lateral root development, flowering, stomatal closure, and defenses against biotic and abiotic stresses (Neill et al., 2002; He et al., 2004; Hong et al., 2008; Wilson et al., 2008; Leitner et al., 2009). NO has also been implicated as a key regulator of senescence and leaf cell death in higher plants (Guo and Crawford, 2005; Zago et al., 2006). While descriptions of NO-mediated processes are accumulating, the details of this small molecule regulates its target signaling networks remain largely unknown (Wang et al., 2010b). To date numerous potential sources for NO production have been described in plants (Gupta et al., 2010), including a nitric oxide synthase (NOS)-like activity (Delledonne et al., 1998; Durner et al., 1998; Besson-Bard et al., 2008), nitrate reductase (NR) (Stohr and Stremlau, 2006; Stoimenova et al., 2007; Seligman et al., 2008), xanthine oxidoreductase (Corpas et al., 2008), and polyamine/hydroxylamine-mediated NO production (Rumer et al., 2009). Archetypal NOS enzymes found in animals (Palmer et al., 1988; Knowles and Moncada, 1994) and also the green algae, *Ostrococcus tauri* (Foresi et al., 2010), catalyze the NADPH-dependent oxidation of L-arginine producing L-citrulline and NO. A similar activity has been reported in higher plants, which is blunted by classical mammalian NOS inhibitors (Delledonne et al., 1998; Durner et al., 1998; Jasid et al., 2006; Vandelle and Delledonne, 2008; Corpas et al., 2009). However, no archetypal NOS encoding genes have so far been identified in higher plants. Another potential source of NO production is NR, which catalyzes the production of this gas by successive reduction of nitrite and nitrate. In Arabidopsis, NR is encoded by two genes, *NIA1* and *NIA2* (Yamasaki and Sakihama, 2000).

It has been showed that NO regulates a number of physiological processes directly by affecting gene transcription (Huang et al., 2002; Wang et al., 2002; Polverari et al.,...
2003; Parani et al., 2004; Shoulars et al., 2008). NO can also regulate specific physiological and developmental processes through interplaying with other small biomolecules. For example, NO can scavenge H$_2$O$_2$ and protects plant cells from damage (Beligni et al., 2002; Crawford and Guo, 2005). It was also reported that NO and H$_2$O$_2$ function in combination to elaborate cell death associated with the hypersensitive response (HR) following pathogen recognition (Delledonne et al., 2001). Furthermore, S-nitrosylation, the addition of an NO moiety to a cysteine thiol to form a S-nitrosothiol (SNO), is emerging as a key redox-based post-translational modification in plants and a pivotal mechanism to convey NO bioactivity. S-nitrosylation may be integral to NO function during a variety of cellular processes. For example, S-nitrosylation of peroxiredoxin IIE during the defense response regulates the antioxidant function of this key enzyme and might contribute to the HR (Romero-Puertas et al., 2007). In animals, SNO formation has been reported to be involved in the regulation of apoptosis at multiple steps (Sawa et al., 1997; Hara et al., 2005; Mannick, 2007). In this context, the S-nitrosylation of GOSPEL (Sen et al., 2009), caspase 3 (Tsang et al., 2009), cyclooxygenase-2 (COX-2) (Carreras and Poderoso, 2007), FLICE (FADD-like ICE) inhibitory protein (FLIP) (Chanvorachote et al., 2005) and apoptosis signal-regulating kinase 1 (ASK1) (Park et al., 2004) are important features of cell death in animals.

The global levels of S-nitrosylation are regulated by S-nitrosoglutathione reductase 1 (GSNOR1) in Arabidopsis (Feechan et al., 2005). Loss-of-function mutations in GSNOR1 increased total cellular SNO content and compromised both non-host and resistance (R) gene-mediated protection and also disabled basal defense responses (Feechan et al., 2005). Further, this line was also perturbed in thermotolerance and responses to paraquat (Lee et al., 2008; Chen et al., 2009).

To generate insights into the potential role of S(NO) in the process of rice leaf cell death and identify associated S-nitrosylated proteins, we conducted a forward genetic screen utilizing a large T-DNA tagged population to identify mutants with perturbed (S)NO homeostasis. This approach uncovered the noel (nitric oxide excess 1) mutant,
which accumulated more (S)NO in comparison to wild type plants. Employing *noe1*, we implicate NR-dependent NO production during rice leaf cell death in response to high light. Furthermore, our findings suggest a function for (S)NO and H$_2$O$_2$ in rice leaf cell death. Through the biotin-switch assay and LC/MS/MS, we identified S-nitrosylated proteins during cell death triggered by high light. These results provide initial insights into the mechanisms underpinning this process in rice leaves.
RESULTS

Identification of noe1

In order to shed light on the role of (S)NO in leaf programmed cell death (PCD), we performed a large-scale screen to identify SNO content-altered mutants using the Saville-Griess assay from our T-DNA insertional mutant population (Ma et al., 2009). To simplify the assay with large amount of samples, we used 96-well plates and a Tecan Infinite M200 (Tecan Group Ltd. Switzerland) to undertake the photometrical measurement at 540 nm. Among the screened 380 mutants that show PCD-like phenotype, 15 mutants with higher SNO content and 4 mutants with lower SNO content were selected for further analysis. One of them, designated noe1 (nitric oxide excess 1), was identified because its SNO content was significantly higher than that of wild type (Fig. 1A). No signs of damage were developed in the shaded parts of the leaves of noe1 grown in the field under natural grown condition (high light, 1600 \( \mu \text{mol.m}^{-2}\text{.s}^{-1} \)) or under low light (400 \( \mu \text{mol.m}^{-2}\text{.s}^{-1} \)) (Supplemental Fig. S1A and S1C). However, leaf damage, characterized by white variegated areas and cell death, developed when noe1 was cultivated under high light (Fig. 1B, 1C, and Supplemental Fig. S1B). When the 10-day-old low light grown noe1 plants were transferred to high light, the obvious leaf bleach and cell death were developed in noe1 plants on the 2nd and 5th day, respectively (Supplemental Fig. S2A). Furthermore, no cell death was observed in noe1 plants cultivated under low light with different temperatures or photoperiods (Supplemental Fig. S2B). By contrast, exposure to high light together with different temperatures (20 °C, 25 °C, and 30 °C) or photoperiods (light to dark, 6-h/18-h, 10-h/14-h, and 14-h/10-h), promoted cell death in all noe1 plants but not in wild-type controls (Supplemental Fig. S2C). These data implied that the observed cell death in noe1 plants is dependent on high light.

Map-Based Cloning of NOE1/OsCATC

Despite being identified from a T-DNA insertion population, the SNO
over-accumulation and cell death phenotypes of noel did not co-segregate with a T-DNA insertion. Thus, the noel line without a T-DNA insertion was isolated by backcrossing and used for further analysis. An F2 mapping population was established using a cross between noel (Japonica) and Minghui 63 (Indica). In the F2 population, we investigated 1,227 individual plants. Among them, 313 plants exhibited the noel mutant phenotype and 914 plants exhibited wild type phenotypes, suggest that noel mutant was controlled by a single recessive gene. Rough mapping delimited the NOE1 locus at about 950 kb on chromosome 3 with genetic markers M2 and M8 (Fig. 1D). Further fine mapping was performed using insertions-deletions (InDels) molecular markers (Supplemental Table I). The NOE1 locus was narrowed down to a 61-kb region between MT19 and MT23 (Fig. 1D). After sequencing all 10 candidate genes within this region, a single-nucleotide G-to-T transition at the 168th position which caused a glutamic acid (GAG) to stop codon (TAG) change was found in LOC_Os03g03910 of noel. Subsequently, we identified another allelic mutant (confirmed by allelic test), noel-2, with a similar aberrant phenotype in leaves and the same higher SNO content compared to wild-type. After sequencing the NOE1 (LOC_Os03g03910) locus, a 21-bp deletion was found, which led to a frame shift mutation (Fig. 1D). NOE1 encodes the rice catalase domain-containing protein and has extreme high sequence similarity with catalase isozyme A (OsCATA) and catalase isozyme B (OsCATB) in rice and three catalase isozymes in Arabidopsis (Supplemental Fig. S3). As in rice genome, there are only three catalase encoding genes, so NOE1 should be OsCATC. Quantitative RT-PCR analysis revealed that the expression of OsCATA, OsCATB, and NOE1/OsCATC varied in different tissues (Fig. 1E). NOE1/OsCATC was mainly expressed in leaf blades, panicles, leaf sheaths, and culms, but expression was extremely low in roots. In contrast, OsCATA was expressed at a high level in leaf blades, panicles, leaf sheaths but weakly expressed in roots and culms. As for OsCATB, high expression was observed in the panicles, leaf sheaths, and culms. In leaves, OsCATA and NOE1/OsCATC were highly expressed but OsCATB was much less abundant (Fig. 1E). In addition, no compensatory induction of OsCATA and OsCATB transcripts was observed in noel plants (Fig. 1F).
results implied that NOE1/OsCATC, but not OsCATA or OsCATB was responsible for the redox homeostasis in leaves. To further validate NOE1/OsCATC, genetic complementation was carried out by introducing the entire cDNA of NOE1/OsCATC into noe1 plants. As expected, the leaf cell death phenotype was not observed in all noe1-C transgenic lines (Fig. 2A). Furthermore, enzyme activity assays showed that the catalase activities of complemented plants were also restored to wild type levels (Fig. 2B). Consequently, H2O2 contents in noe1-C plants were also decreased to that of wild type (Fig. 2C), as well as the content of SNO (Fig. 2D). Consistent with the impairment of NOE1/OsCATC, total catalase activity in noe1 plants was reduced dramatically to about 30% of that of wild type (Fig. 2B), and more H2O2 and cell death accumulated (Fig. 2C and Supplemental Fig. S4). Addition of 200 μM CAT (catalase, a H2O2 scavenger), the leaf cell death in noe1 plants did not develop at the 2nd or 5th day under high light (Supplemental Fig. S5A and S5C). Furthermore, cell death in noe1 suspension cells was reduced from 53.9% to 16.6%, after treatment with 200 μM CAT (Supplemental Fig. S5E). These data suggest that leaf cell death in noe1 may be caused by H2O2 over-accumulation. Taken together, loss-of-function mutations in NOE1/OsCATC are responsible for the phenotypes displayed in the noe1 mutants.

H2O2 Stimulates NO Production

Labeling NO with the cell-permeable, NO-specific fluorescent probe 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate (DAF-FM DA) and imaging by confocal microscopy revealed that NO content in noe1 suspension cells was about 2.3-fold greater than that of wild type (Fig. 3A and 3B). To elucidate the relationship between H2O2 and NO, a treatment of wild type and noe1 suspension cells with 1 mM H2O2 was performed and DAF-FM DA analysis showed that H2O2 induced NO production significantly (Fig. 3A and 3B). The seedlings of wild type and noe1 grown under low light for 10 days were transferred to high light, then H2O2 and SNO contents were measured at the 6th, 8th, and 10th day under low light and each day under high light. When grown under low light, there was no significant difference
between wild type and noel plants in relation to H\textsubscript{2}O\textsubscript{2} and SNO contents, which increased gradually with time (Supplemental Fig. S6). After transfer to high light, however, H\textsubscript{2}O\textsubscript{2} and SNO were produced more rapidly within the first day in noel plants as compared with wild type plants (Supplemental Fig. S6). These findings indicated that the rapid increase of H\textsubscript{2}O\textsubscript{2} and SNO detected in noel plants is dependent on high light. Further detailed analysis was made every two hours on the first day under high light. The rapid production of H\textsubscript{2}O\textsubscript{2} and SNO in noel occurred at the 2nd and 8th hour, respectively (Fig. 3C and 3D). In addition, treatment with different concentration of CAT revealed that the generation of SNO was inhibited in a dose-dependent manner (Fig. 3E). In contrast, H\textsubscript{2}O\textsubscript{2} content was not altered after treatment of high light grown noel plants with 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO, an NO scavenger) (Fig. 3F). These results suggested that H\textsubscript{2}O\textsubscript{2} stimulated NO production may cause the increased SNO levels in leaves of noel plants under high light.

### H\textsubscript{2}O\textsubscript{2}-Induced NR-dependent Generation of NO

To elucidate the main source responsible for NO production in noel plants under high light, the activities of nitric oxide synthase (NOS) and nitrate reductase (NR) were measured. No difference in NOS activity was observed between wild type and noel plants (Fig. 4A). NR activity in noel plants, however, was approximately twice that of wild type (Fig. 4B). Quantitative real-time PCR analysis revealed that the expression of OsNIA1 and OsNIA2 increased 3- and 9-fold respectively in noel plants (Fig. 4C and 4D), in line with the increase of NR activity. As a result, the content of the NR substrate, nitrate, was decreased in noel plants (Fig. 4E). To further confirm NO was mainly produced from NR activity, tungstate (a NR inhibitor) and L-NMMA (a NOS inhibitor) were used and SNO contents were monitored. The content of SNOs was inhibited in a dose-dependent manner when noel seedlings treated with tungstate, while no change in SNO content was observed in noel seedlings treated with L-NMMA (Fig. 4F). These results implied that the excess NO in noel was mainly generated from NR. Presumably, this increased NO concentration subsequently
resulted in greater SNO levels.

**NO is Required for H$_2$O$_2$-Induced Leaf Cell Death**

To identify the role of NO in H$_2$O$_2$-induced leaf cell death modulated by light (Supplemental Fig. S1 and S2), treatments of wild type and noel seedlings with PTIO and SNP (sodium nitroprusside, an NO donor), both alone and in combination were carried out. After transferring the 10-day-old low light grown noel plants to high light, obvious leaf bleaching and cell death formation occurred on the 2nd and 5th day, respectively (Fig. 5A and 5C). Addition of 200 μM PTIO which depletes NO, resulted in significantly reduced leaf cell death (Fig. 5). By contrast, leaf cell death in noel plants was promoted following treatment with 2.5 mM SNP (Fig. 5). Cell death could also be triggered in wild-type plants following addition of 2.5 mM SNP for 5 days (Fig. 5C and 5D), demonstrating a requirement for NO in the process of cell death. Similar treatments were performed with dark grown rice suspension cells, in which cell death can be accurately measured. Results showed that the relative cell death ratio in noel suspension cells increased from 14.2% to 54.5% when exposed to low light (400 μmol.m$^{-2}$·s$^{-1}$) for 1 day (Fig. 5E). After addition of 200 μM PTIO, the ratio of cell death was reduced to 19.7% when exposed to low light for 1 day, this level was similar to that found in wild type cells (15.4%). When cultured with 2.5 mM SNP under low light for 1 day, the relative cell death ratio in wild type and noel suspension cells increased to 42.4% and 73.1% respectively, significantly greater than control values (wild type and noel: 15.0% and 54.5% respectively) (Fig. 5E). These results suggest that NO is a pivotal mediator for H$_2$O$_2$-induced leaf cell death in rice.

**Protein S-nitrosylation is involved in H$_2$O$_2$-Induced Leaf Cell Death**

It is known that protein S-nitrosylation is a fundamental mechanism to transduce NO bioactivity (Besson-Bard et al., 2008) and GSNOR regulates global levels of S-nitrosylation (Feechan et al., 2005). OsGSNOR is encoded by a single gene in rice which has very high sequence similarity with AtGSNOR (Supplemental Fig. S7). To investigate the potential role of *OsGSNOR* in leaf cell death, *OsGSNOR*
over-expression (GSNOR-O noe1) or knockdown (GSNOR-R noe1) transgenic plants in the noe1 background were generated (Fig. 6A). GSNOR-O noe1 plants with increased GSNOR activity and GSNOR-R noe1 plants with reduced GSNOR activity were selected for further analysis (Fig. 6B). Employing the Saville-Griess assay, lower SNO content was found in GSNOR-O noe1 plants relative to noe1 lines. In contrast, GSNOR-R noe1 plants exhibited an increased level of these redox molecules (Fig. 6C). These data are consistent with those from Arabidopsis, which suggested that GSNOR controls the total level of SNOs in cells (Feechan et al., 2005). The biotin-switch assay, labeling of S-nitrosylated proteins with a biotin moiety specifically on S-nitrosylated cysteines (Jaffrey and Snyder, 2001), further confirmed these results (Fig. 6D). When cultivated under high light, there was no difference in phenotype between either the GSNOR-O noe1 or GSNOR-R noe1 transgenic lines relative to that of noe1 plants (data not shown). However, when these lines were grown under low light for 10 days and then subsequently exposed to sustained H2O2 synthesis (generated by glucose/glucose oxidase system, which could moderately release H2O2) for 3 days, leaf cell death in GSNOR-O noe1 plants was less than that observed in noe1 plants (Fig. 6E). Collectively, these findings imply that protein S-nitrosylation is involved in H2O2-induced leaf cell death.

Proteomic Identification of S-Nitrosylated Proteins

To identify possible candidates for S-nitrosylation integral to H2O2-induced leaf cell death, the biotin-switch assay and LC/MS/MS were performed. Total protein, extracted from wild type and noe1 leaves grown under low light for 10 days and subsequently transferred to high light for 2 days, were blocked with MMTS and labeled with Biotin-HPDP (Wang et al., 2009), after precipitation with acetone, the re-suspended mixture was firstly digested with trypsin before purification with affinity chromatography to harvest the biotin-labelled peptides, then S-nitrosylated peptides were judged by LC/MS/MS. 73 and 100 proteins were identified from wild type and noe1 plants, respectively, among them 52 proteins common to both wild type and noe1 plants (Fig.7A). Proteins identified here are involved in different processes...
including general metabolism, environmental adaption, genetic information processing, and redox regulation (Fig. 7B and Supplemental Fig. S9). Almost half the proteins identified were reported previously as being subject to \( S \)-nitrosylation (supplemental Table. VII, VIII, and IX), indicating the reliability of these results. Among the proteins identified in \( noe1 \) plants, approximately 37.5% functioned in general metabolism and 21% in genetic information processing (Fig. 7B). Proteins related to environmental adaption and redox homeostasis accounted for 10% and 14% respectively (Fig. 7B). Among the 7 redox-related \( S \)-nitrosylated proteins identified in \( noe1 \), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and thioredoxin (TRX) were previously reported to be involved in cell death in animals and their function was linked with their \( S \)-nitrosylation (Sumbayev, 2003; Hara et al., 2005) (Table 1). These results further implied that protein \( S \)-nitrosylation is involved in \( H_2O_2 \)-induced leaf cell death.
DISCUSSION

NO and ROS synthesis is a routine requirement for plant cells to undergo PCD, however, in some contexts evidence has been presented that either can induce HR independently of the other (Clarke et al., 2000). The emerging data also suggest that significant cross-talk occurs between NO and ROS (Delledonne et al., 2001), although the molecular details of this interchange remain undefined. Here, we identified a rice NO accumulation mutant, noe1, following a map-based cloning approach. NOE1 was found to encode rice catalase OsCATC. Further analysis revealed that NOE1/OsCATC is the ortholog of AtCAT2 (Iwamoto et al., 2000; Zimmermann et al., 2006). A previous report showed that cell death in cat2 plants grown in long days was linked to photoperiod not light intensities (Queval et al., 2007). However, this data contrasted with our findings with noe1 plants. When exposed to high light, the lack of leaf catalase activity in noe1 plants resulted in an accumulation of H₂O₂ which further induced NO production. Conversely, reduction of intracellular NO levels, using the scavenger PTIO or the levels of SNO by over-expression of OsGSNOR, alleviated leaf cell death triggered by H₂O₂ in noe1 plants. In addition, treatment of noe1 plants and OsGSNOR RNAi transgenic lines with the NO donor, SNP, promoted cell death. Taken together, these results imply that NO and SNO are important mediators of light-dependent leaf cell death in rice. Previous work has also shown that NO acts as a pivotal positive mediator in cadmium (Cd²⁺)-induced PCD in suspension cell cultures (De Michele et al., 2009), leaf senescence (Carimi et al., 2005), and the HR (Zaninotto et al., 2006). Additionally, cell death in dicotyledonous soybean suspension cultures has been reported to be regulated by the intracellular NO:ROS ratio (Delledonne et al., 2001). In contrast, it was suggested that NO acts as an antagonist to delay PCD in barley aleurone layers and leaf senescence in Arabidopsis (Beligni et al., 2002; Guo and Crawford, 2005; Mishina et al., 2007). These discrepancies for the role of NO in cell death might be due to the differences in plant species, redox state, and growth conditions.
The molecular mechanism(s) of NO generation in response to various stimuli still remain controversial. However, the addition of tungstate (an NR inhibitor) dramatically blunted NO accumulation in rice following exposure to high light, suggesting that increased NO accumulation in noel plants in response to this stimulus was predominantly generated from NR. Moreover, the increase of NR activity in noel plants under high light correlated with the up-regulation of two NR-encoding genes, OsNIA1 and OsNIA2. In Arabidopsis, increased NR activity in response to light, nitrate, and sucrose was also congruent with increased expression of NIA1 and NIA2 (Cheng et al., 1992; Campbell, 1999). Thus, these observations provide further circumstantial evidence of a role for NR in NO synthesis in response to high light.

Interestingly, promoter analysis also revealed a 28-bp motif in the promoter region of OsNIA1 and OsNIA2, which is similar to the redox responsive motif identified in OsSODCC1, OsTRXH, and OsGRX (Tsukamoto et al., 2005). Thus, increasing H2O2 levels following exposure to high light might increase NR expression through this redox-responsive element. Furthermore, several reports have suggested that NR activity can be modulated at the post-transcriptional level. For example, H2O2 is required for the activation of mitogen-activated protein kinase 6 (MPK6), which can subsequently increase NR activity by phosphorylation of NIA2 at Ser-627 (Wang et al., 2010a). Also, NIA2 can be phosphorylated by a calcium-dependent protein kinase at Ser-534 in the hinge 1 region in response to light and other environmental signals (Su et al., 1996). Therefore, increased cellular H2O2 concentrations following exposure to high light might also increase NR activity through phosphorylation of either Ser-627 or Ser-534.

As S-nitrosylation is a major route for the transduction of NO bioactivity, we identified 73 and 100 S-nitrosylated proteins from wild type and noel plants respectively, by employing the biotin-switch assay and LC/MS/MS. Among these proteins, there were 52 S-nitrosylated proteins from both wild type and noel plants. These proteins are involved in a wide array of cellular activities, such as genetic information processing, general metabolism, environmental adaption, and redox
homeostasis. Both GAPDH and TRX, which were reported to be involved in animal cell death (Sumbayev, 2003; Hara et al., 2005), were only S-nitrosylated in noe1 plants (Table I). In mammals, GADPH is S-nitrosylated at its active site, Cys-150, which promotes its association with the E3-ligase, Siah1, resulting in nuclear translocation of the SNO-GAPDH–Siah1 complex (Mannick, 2007). Within the nucleus, SNO-GAPDH stabilizes Siah1 and facilitates the ubiquitination and degradation of nuclear proteins (Hara et al., 2005), which promotes cell death (Foster et al., 2009; Tristan et al., 2011). GAPDHs also play a central role in the carbon economy of plant cells and higher plants possess four distinct isoforms of this protein (Hajirezaei et al., 2006; Munoz-Bertomeu et al., 2009; Munoz-Bertomeu et al., 2010). A knock-out line of cytosolic GAPDH (GAPC) shows reduced levels of oxygen uptake and ATP but increased ROS accumulation and also a higher density of trichomes (Rius et al., 2006). TRX is a key modulator of redox status (Vieira Dos Santos and Rey, 2006). During nitrosative stress, S-nitrosylation of TRX, presumed to be S-nitrosylated at Cys-32 or Cys-35 within the active site, promotes apoptosis presumably by inhibiting the oxidoreductase function of this enzyme and by facilitating the release of sequestered apoptosis signal-regulating kinase-1 (ASK1) (Sumbayev, 2003; Hess et al., 2005). ASK1 is a mitogen-activated protein kinase (MAPK) that specifically activates the Jun N-terminal kinase (JNK) and p38 MAPK signalling networks and is integral to tissue necrosis factor (TNF) α-induced apoptosis (Hess et al., 2005). Thus, S-nitrosylation of both GAPDH and TRX in noe1 but not wild type plants parallels the development of cell death in animal systems.

Ascorbate peroxidase (Vacca et al., 2004), glutaredoxin (Saeed et al., 2010), acyl carrier protein (Feng et al., 2009), phosphomannomutase (Hoeberichts et al., 2008), fructose-bisphosphate aldolase isozyme (Yao et al., 2004), triose phosphate isomerase (Gnerer et al., 2006), pyruvate kinase (Stetak et al., 2007), acidic 27 kDa endochitinase (Shin et al., 2009), and lactate/malate dehydrogenase (Matthews et al., 2004) have all previously been reported to be involved in programmed cellular execution (Table I, supplemental table. IV). However, the molecular mechanisms
associated with the functions of these proteins in cell death remain to be established. Our findings imply that all of these proteins may also be S-nitrosylated in the rice noe1 mutant. Interestingly, although noe1 plants were not subjected to any pathogen challenge, a pathogenesis-related Bet v I family protein and PR1b were both S-nitrosylated (supplemental table. V), suggesting this modification might regulate the activity of these gene products. Ribosomal related proteins (supplemental table. VI) were also S-nitrosylated, indicating that this modification might also be involved in the regulation of protein translation. Furthermore, an elongation factor was also found to be a target for S-nitrosylation, suggesting NO may control protein synthesis in response to H$_2$O$_2$ (Shenton and Grant, 2003; Lindermayr et al., 2005).

Despite a central role for GSNOR in SNO homeostasis, information on the regulation of this enzyme is limited (Besson-Bard et al., 2008). Constitutively increased GSNOR activity in Arabidopsis atgsnor1-1 and atgsnor1-2 mutants correlated with elevated AtGSNOR1 mRNA levels (Feechan et al., 2005). However, GSNOR protein abundance, rather than the magnitude of transcripts was promoted by paraquat (Chen et al., 2009). In contrast, neither AtGSNOR1 protein levels nor transcript levels were modulated during heat acclimation, suggesting that this stimulus may control GSNOR function at the post-transcriptional level (Larkindale and Vierling, 2008; Lee et al., 2008). Our data suggest that OsGSNOR transcript accumulation in noe1 plants grown under high light levels was only one third that of wild type (Fig. 6A). Simultaneously, the activity of this enzyme in noe1 plants was reduced to 43% of the value determined in wild type plants. Collectively, these data imply OsGSNOR function during high light intensities might be controlled at the transcriptional level. However, other mechanisms including post-translational modification might also be significant. In this context, S-nitrosylation would be a strong candidate and GSNOR has indeed been found to be S-nitrosylated in the acclimation of citrus plants to salinity (Tanou et al., 2009). GSNOR loss-of-function mutations in mice, Arabidopsis, and yeast result in increased SNO levels (Liu et al., 2001; Liu et al., 2004; Feechan et al., 2005; Rusterucci et al., 2007). Here, over-expression of OsGSNOR in noe1 plants reduced
SNO levels, consistent with a key role for this enzyme in SNO homeostasis. Moreover, our results show that no change in H$_2$O$_2$ content occurred in either GSNOR-O noe1 or GSNOR-R noe1 transgenic lines (Supplemental Fig. S8), suggesting that NO might function downstream of H$_2$O$_2$ in light-driven leaf cell death in rice.

Collectively, we have identified an NO accumulation mutant noe1 in rice. Map-based cloning revealed that NOE1 encoded OsCATC. noe1 resulted in an increase of hydrogen peroxide (H$_2$O$_2$) in the leaves, which consequently promoted NO production via activation of nitrate reductase (NR). Our data imply that NO and SNO are important mediators of light-dependent leaf cell death in rice. Further, we have uncovered a series of S-nitrosylated proteins in both noe1 and wild type plants, which may help to reveal the role of this post-translational modification in the control of light-mediated leaf cell death in rice.
MATERIALS AND METHODS

Plant Growth and Sampling
Rice (*Oryza sativa* L.) plants were cultivated in the experimental field of the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences in Beijing. Treatments were performed using the 10-day-old rice seedlings. Seeds of wild type and *noel* were submerged in water for 2 days at 37 °C, then the uniformly germinated seeds cultured in 96-well plates supplied with 1/2 strength Murashige and Skoog (1/2 MS) basal medium (pH 5.8) for 10 days in the growth chamber at 30 °C for 14 h (low light, 400 μmol.m⁻².s⁻¹) and 28 °C for 10 h (dark). After the seedlings were transferred to high light (1600 μmol.m⁻².s⁻¹), leaf samples were taken on the 2nd and 5th day to measure the SNO and H₂O₂ content. The chemicals used to treat the 10-day-old seedlings, SNP (an NO donor), PTIO (an NO scavenger), CAT (catalase, H₂O₂ scavenger), tungstate (an NR inhibitor), and L-NMMA (an NOS inhibitor) were added with 200 ml 1/2 MS media. All chemicals were purchased from Sigma-Aldrich. *GSNOR-O noe1* and *GSNOR-R noe1* transgenic lines and control were cultivated under low light for 10 days and treated with 200 μM PTIO, 2.5 mM SNP and H₂O₂ (about 400 μmol·L⁻¹, generated by 2.5 mM glucose plus 2.5U/ml glucose oxidase (Kim et al., 2010)) for 3 days. All experiments were repeated three times.

Map-based Cloning of *NOE1/OsCATC*
To map the *NOE1* gene, a F₂ population derived from a cross between *noel* (*Japonica*) and Minghui63 (*Indica*) was constructed. The genomic DNA from 313 F₂ progenies with leaf cell death phenotype was extracted with a modified CTAB method described previously (Mou et al., 2000). To fine-map *NOE1*, some polymorphic InDel markers were generated based on the different sequence between *Japonica* and *Indica*. The locus was finally defined to a 61-kb region and all the 10 candidate genes in this region were sequenced and analyzed using SeqMan within Lasergene version 5.0 software (DNASTAR). Primer sequences are listed in Supplemental Table I.
Real-Time PCR Analysis

Total RNA was extracted using the TRIzol kit according to the user manual (GenStar, China) (Tong et al., 2009). Two microgram of total RNA treated with DNase I and was used for cDNA synthesis with a reverse transcription kit (Promega, USA). Real-time PCR experiments were performed using gene-specific primers in a total volume of 20 μl containing 0.3 μl dNTP, 2 μl 10×Taq buffer, 0.2 μl Taq enzyme, 0.5 μl 40× SYBR Green Master mix (Applied Biosystems), 1.5 μl of 1 mM gene-specific primers and 1 μl cDNA on a C1000™ Thermal Cycler (CFX96 real-time system, BioRad, USA) as described before (Yang et al., 2009). The rice 18S gene was used as the internal control. Quantification consisted of at least three independent replicates. The primer sequences used are listed in Supplemental Table II.

Complementation of noe1 and OsGSNOR Vector Construction

The binary vector pXQAct, a derivative of pCambia 2300, carrying the rice Actin1 promoter and the OCS terminator, was used for plant transformation (Sun et al., 2011). For complementation of the noe1 mutant, the 1,640 bp full-length cDNA of NOE1/OsCATC with XbaI site amplified from Nipponbare cDNA was ligated to the pXQAct, resulting in pnoe1-C vector. The 1,266 bp full-length cDNA of OsGSNOR amplified was digested with SmaI and SalI and subsequently ligated into the pXQAct, resulting in pGSNOR-O vector. To generate the RNAi construct pGSNOR-R, a 318 bp fragment was amplified from OsGSNOR cDNA and was sequentially cloned into XhoI/BglII and BamHI/SalI sites of pUCC-RNAi vector to target the gene in both the sense and antisense orientations (Luo et al., 2005). The whole-stem loop fragment was further cloned into pXQAct, yielding the binary GSNOR RNAi vector. These plasmids were introduced into Agrobacterium tumefaciens AGL-1 by freeze-thaw transformation. Consequently the mutant noe1 callus was transformed by an Agrobacterium-mediated method as described previously (Liu et al., 2007). The primer sequences used for vector construction are listed in Supplemental Table III.

Cell Death Analysis
Relative cell death of suspension cells was assayed as described previously with some modifications (Delledonne et al., 2001). Briefly, after 24 h treatments with CAT, PTIO, SNP, and SNP+PTIO under low light (400 μmol.m$^{-2}$.s$^{-1}$), the rice suspension cells were stained with 0.05% Evan’s blue (Sigma-Aldrich) for 15 min. The excess dye was removed by extensive washing and dye bound to dead cells was solubilized in 50% (v/v) methanol/1% SDS in a volume of 20 ml for 30 min at 50°C and quantified by absorbance at 595 nm. The OD$_{595}$ of rice cell suspensions saturated with methanol for 15 min as the total killed cell death. The relative cell death is expressed as a ratio of treatment OD$_{595}$ to total killed cell death OD$_{595}$. The relative cell death of wild type and noe1 seedlings leaves were analyzed using the software Adobe Photoshop CS and expressed as percentage of cell death region to total leaf region.

**Histochemical Detection**

H$_2$O$_2$ was detected by DAB staining as described previously with some modification (Thordal Christensen et al., 1997). The fully expanded leaves of 2-month-old wild type and noe1 plants were detached and infiltrated with the 0.1% DAB solution. The sampled leaves were placed in a growth chamber for 5 h at 28 °C and cleared in boiling ethanol (95%) for 10 min. The chlorophyll was removed by incubating in 95% (v/v) ethanol overnight before photographing.

Superoxide (O$_2^-$) accumulation in rice leaves was visualized by 0.1% NBT staining as described (Fang et al., 2008). The fully-expanded leaves of 2-month-old wild type and noe1 plants were stained with NBT solution. After staining overnight, the chlorophyll was removed by incubating in 95% (v/v) ethanol overnight.

Dead cells were stained using detached leaves by a method modified by Waspi (Waspi et al., 2001). Leaves of 2-month-old wild type and noe1 plants were submerged in Trypan blue solution at 70 °C for 10 min, then heated in boiling water for 2 min and left staining overnight. After destaining in chloral hydrate solution (25 g chloral hydrate in 10 ml of H$_2$O) for 3 days, samples were equilibrated with 70% glycerol for microphotograph.
Malondialdehyde (MDA) Content Assay

Malondialdehyde (MDA) was regarded as an indicator of lipid peroxidation and cell death. The content of MDA was determined with a MDA assay kit (Beyotime, China) with some modifications (Qian et al., 2010). Briefly, leaves (0.02 g fresh weight) was homogenized in 2 ml of phosphate buffer (pH 7.8) containing 1% PVP and centrifuged at 2,500 g for 10 min. MDA in the supernatant was determined according to the reaction with thiobarbituric acid and measured the absorbance at 450, 532, and 600 nm. According to the formula $6.45 \times (\text{OD}_{532} - \text{OD}_{600}) - 0.56 \times \text{OD}_{450}$, the MDA content was expressed as nM MDA per gram fresh weight.

Quantitative Measurement of H\textsubscript{2}O\textsubscript{2} Content and Catalase Activity

The measurement of H\textsubscript{2}O\textsubscript{2} production was performed by extracting H\textsubscript{2}O\textsubscript{2} from leaves according to a previously described method (Huang et al., 2009) and quantifying the molecule with an Amplex Red Hydrogen Peroxidase Assay Kit (Molecular Probes, Invitrogen) according to the manufacturer’s instruction. Catalase activity was carried out with a Catalase Assay Kit (Beyotime, China) according to the manufacturer’s instructions.

Measurement of Ion Leakage

The measurement of ion leakage was performed according to previously described method (Woo et al., 2001). Five different leaf discs from wild type and noel\textsubscript{1} plants were placed into a 100 ml beaker containing 40 ml distilled deionized water. After shaking at 120 rpm for 3 h, the conductivity (C1) was measured. Then the discs were boiled for 10 min and shaken for 1 h and measured the conductivity (C2) again. The ion leakage was calculated according to the formula $(C1/C2) \times 100\%$.

Saville-Griess Assay

SNO content was measured using the Saville-Griess assay as described with minor modification (Feechan et al., 2005). In the Saville–Griess assay, S-NO bonds was displaced by mercuric chloride reacts with sulfanilamide. The resulting diazonium salt
is coupled with the aromatic amine N-(1-naphthyl)-ethylenediamine to form an intensely colored azo dye that can be measured at 540 nm (Feechan et al., 2005; King et al., 2005). Briefly, fine powder of plant tissues from liquid nitrogen was lysed in 600 µl extraction buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl) containing 1 mM protease inhibitor PMSF and incubated on ice for 20 min. After centrifuge 10,000 rpm for 15 min at 4 °C, 160 µl supernatant clear cell lysate was incubated with same volume of 1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine with or without the addition of 3.75 mM HgCl2 for 20 min in dark. Then SNO content was measured photometrically at 540 nm using 96-well plates with Tecan Infinite M200 (Tecan, Switzerland). The SNO content was calculated according to the absorption at 540 nm and GSNO concentration standard curve (Feechan et al., 2005).

**Determination of Endogenous NO Content of Suspension Cells with DAF-FM DA**

Endogenous NO level of suspension cells was detected by imaging the cell-permeable, NO-specific fluorescent probe 4-amino-5-methylamino-2’,7’-difluorofluorescein diacetate (DAF-FM DA, Invitrogen) by confocal microscopy with some modifications (Zhao et al., 2007). Wild type and noe1 suspension cells (untreated or treated with 1 mM H2O2 for 30 min at 28 °C) were incubated with 100 µM Tris-HCl (pH 7.6) containing 5 µM DAF-FM DA at 28 °C for 40 min. Then washed the cells three times with fresh Tris-HCl (100 µM, pH 7.6) to remove excess probe and incubate for an additional 20 min to allow complete de-esterification of the intracellular diacetates. The incubated cells were visualized using a laser confocal scanning microscope (Leica TCS SP5). Excitation/emission wavelength were 488/515 nm. Data was presented as mean pixel intensities. Fifty to one hundred suspension cells are observed per treatment for three independent replicates.

**Determination of NOS, NR Activity, and Nitrate Content**

The activity of NOS and NR were determined with an NOS Assay Kit and Nitrate/Nitrite Assay Kit (Beyotime, China) with some modifications (Xiong et al.,
2009). Total protein was extracted using the buffer containing 100 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.1% Triton X-100, 0.5 mM PMSF, 20 µM FAD, 25 µM leupeptin, 5 µM Na₃MoO₄, and 1% PVP. Then the NOS and NR activity were measured according to the manufacturer’s instructions. Nitrate content were measured using a Siever Nitric Oxide Analyzer (NOA™) 280i (Siever, GE Analytical Instruments) according to the manual instructions.

**GS-FDH GSNOR Enzyme Activity Assay**

GSNOR enzyme activity was measured spectrophotometrically at 340 nm at 25°C by monitoring the decomposition of NADH as described before (Durner et al., 1998). GS-FDH activity was determined by incubating 100 µg of protein in 300 µl of assay mixture that contained 20 mM Tris-HCl (pH 8.0), 0.2 mM NADH, and 0.5 mM EDTA. The reaction was started by adding GSNO (ALEXIS Biochemicals) at a final concentration of 400 µM.

**Protein Extractions**

Protein extractions were performed according to a previously described method (Jaffrey and Snyder, 2001). In brief, leaf tissues (200 mg) were ground in liquid nitrogen using a mortar and pestle. Soluble proteins were extracted from the resulting powder at 4°C in 1 ml HEN buffer (250 mM HEPES-NaOH pH 7.7, 1 mM EDTA, 0.1 mM Neocuproine, and 10 mM PMSF) in the dark. The total extracts were incubated on ice for 20 min and centrifuged at 10,000 rpm for 15 min at 4°C.

**Biotin-switch Assay and Isolation of Biotinylated Proteins**

Biotin-switch assay was performed as described previously (Wang et al., 2009). Three milligram of soluble total protein were used for assay. After Biotin-HPDP labeling step, around 30 µg of labeled soluble proteins were used for western blot according to the standard protocol. Add two volumes of -20 °C acetone to the rest samples and incubate for 20 min at -20°C and then centrifuge at a minimum of 2,000 g for 10 min at 4°C. The supernatant was discarded to remove biotin-HPDP. Then gently rinse the
wall of the tube and the surface of the pellets with -20 °C acetone to remove traces of biotin-HPDP. Resuspend the pellets in 0.1 ml of 25mM NH₄HCO₃ buffer per mg of protein in the initial protein sample and digested with trypsin (sequencing grade modified trypsin, Promega Corporation, USA) with protease protein ratio 1:20 (w/w) at 4 °C overnight. Then add two volumes of Neutralization buffer (20 mM HEPES-NaOH pH 7.7, 100 mM NaCl, 1 mM EDTA, and 0.5% Triton X-100). Add 15 µl of packed streptavidin-agarose resin per mg of protein used in the initial protein sample to purify biotinylated proteins. Incubate the proteins with resin for 1 h at room temperature with continuous shaking. Wash the resin five times with 10 volumes of Neutralization buffer plus 600 mM NaCl and centrifuge at 200 g for 5 seconds at room temperature between each wash. Incubate the resin with Elution buffer (20 mM HEPES-NaOH pH 7.7, 100 mM NaCl, 1 mM EDTA, and 100 mM mercaptoethanol) to recover the bound proteins. Purified protein extracts were identified by LC/MS/MS analysis to find S-nitrosylated proteins.

According to previous report (Wang et al., 2009), LC/MS/MS experiments were carried out on a Thermo LTQ Orbitrap XL instrument (Thermo Electron Corporation, USA) equipped with a house made source and an Eksigent 2D nanoLC system (Eksigent Technologies, USA). Sample analyses were performed using an in-house made reverse phase C18 capillary column (15 cm × 100 μm I.D.). The peptides were sequentially eluted with a gradient of 0 to 80% of buffer B (acetonitrile with 0.5% formic acid) in buffer A (water with 0.5% formic acid) at a flow rate of 400 nl/min. The electrospray source parameters were 2.0 kV of electrospray voltage, the range of m/z was from 300 to 1,800, normalized collision energy of MS/MS was 35%. The data were analyzed with Bioworks 3.31 and Sequest 2.8, results were filtered by Xcorr (the cross-correlation value from the search) +1>1.9, +2>2.5, +3>3.75, sp>500, Δcn (the Delta Correlation value) >0.1, Rsp≤5. The pathways of proteins exert their roles were performed by searching in KEGG (http://www.genome.jp/dbget-bin/www_bfind?O.sativa).
ACKNOWLEDGEMENTS

We thank Dr. Jeum Kyu Hong from Jinju National University, South Korea for valuable advice and critical reading of the manuscript. This work was supported by grants from National Natural Sciences Foundation of China (Grant No. 31171514, 30600407, 30825029, 30921061), Ministry of Science and Technology of China (Grant No. 2009CB118506) and an international exchange grant provided by the National Natural Science Foundation of China and the Royal Society of Edinburgh (Grant No. 30811130222).
SUPPLEMENTAL DATA

**Supplemental Figure S1.** Leaf cell death in *noe1* is dependent on high light

**Supplemental Figure S2.** Leaf cell death in *noe1* is dependent on light intensity

**Supplemental Figure S3.** NOE1/OsCATC sequence alignment with other catalase proteins from rice and Arabidopsis

**Supplemental Figure S4.** More H$_2$O$_2$ accumulated and cell death occurred in *noe1*

**Supplemental Figure S5.** H$_2$O$_2$-induced leaf cell death in *noe1*

**Supplemental Figure S6.** H$_2$O$_2$ and SNO levels in wild type (WT) and *noe1* seedlings at different time-points.

**Supplemental Figure S7.** Sequence alignment of GSNOR from rice and Arabidopsis

**Supplemental Figure S8.** H$_2$O$_2$ contents in 4-month-old wild type (WT), *noe1*, and *OsGSNOR* transgenic lines grown under high light (1600 μmol.m$^{-2}$.s$^{-1}$)

**Supplemental Figure S9.** Functional categorization of S-nitrosylated proteins identified from wild type and *noe1* plants

**Supplemental Table I.** Primers used for cloning

**Supplemental Table II.** Primers used for qRT-PCR

**Supplemental Table III.** Primers used for vector construction

**Supplemental Table IV.** S-nitrosylated proteins that reported to trigger cell death identified from *noe1* plants

**Supplemental Table V.** S-nitrosylated proteins subjected to environmental adaption identified from *noe1* plants

**Supplemental Table VI.** S-nitrosylated proteins related to ribosome identified from *noe1* plants

**Supplemental Table VII.** S-nitrosylated proteins identified from both wild type and *noe1* plants

**Supplemental Table VIII.** S-nitrosylated proteins identified only from *noe1* plants

**Supplemental Table IX.** S-nitrosylated proteins identified only from wild type plants
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FIGURE LEGENDS.

Figure 1. Phenotypes of noe1 and map-based cloning of NOE1/OsCATC. SNO content (A) and phenotypes (B and C) of 3-month-old wild type (WT) and noe1 plants grown under high light (1,600 μmol.m⁻².s⁻¹). D, Map-based cloning of NOE1/OsCATC. The NOE1/OsCATC gene was mapped in the interval of MT2 and MT8 on short arm of chromosome 3 and was delimited to a 61-kb region with 10 candidate genes between the MT19 and MT23 marker, the mutated sites of two distinct allelic mutant were shown in the gene LOC_Os03g03910. E, Expression patterns of OsCATA, OsCATB, and NOE1/OsCATC in different tissues analyzed by quantitative RT-PCR. F, Expression levels of OsCATA, OsCATB, and NOE1/OsCATC in wild type and noe1 leaves. These data was obtained from three independent replicates. * P< 0.05 (t test).

Figure 2. Phenotypes of noe1 were complemented by NOE1/OsCATC. A, phenotypic comparison among 3-month-old wild type (WT), noe1, and complemented transgenic lines noe1-C. B, C, and D, total extractable leaf catalase activities, H₂O₂ and SNO contents in 3-month-old wild type, noe1, and noe1-C plants grown under high light (1,600 μmol.m⁻².s⁻¹). These data were obtained from three independent replicates.

Figure 3. Accumulated H₂O₂ stimulated NO production and caused the increase of SNO level. A, NO level (A) and relative fluorescence intensity (B) (control or with treatment of 1 mM H₂O₂ or 200 μM PTIO for 30 min at 28 °C) in wild type (WT) and noe1 suspension cells was monitored by labeling NO using the specific NO dye DAF-FM DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate). H₂O₂ (C) and SNO (D) contents in wild type and noe1 seedlings at 2 h interval on the first day following exposure to high light. E, SNO content in wild type and noe1 seedlings treated with 100, 200, and 400 μM CAT (catalase, a H₂O₂ scavenger) for 2 days under high light. F, H₂O₂ content in wild type and noe1 seedlings treated with 50, 100, and
200 µM PTIO for 2 days under high light. Both 10-day-old wild type and noel seedlings cultivated under low light (LL, 400 µmol.m⁻².s⁻¹) were transferred to high light (HL, 1,600 µmol.m⁻².s⁻¹). These data were obtained from three independent replicates. ** P< 0.01 (t test).

**Figure 4.** Nitrate reductase (NR) was mainly responsible for the S(NO) accumulation in noel. NOS (A) and NR (B) activity, relative expression levels of OsNIA1 (C) and OsNIA2 (D) by quantitative RT-PCR (WT, set as 1.0), and nitrate content (E) in wild type (WT) and noel plants. F. Effects of tungstate (an NR inhibitor) and L-NMMA (an NOS inhibitor) on endogenous NO production in wild type and noel plants. Both 10-day-old wild type and noel seedlings cultivated under low light (400 µmol.m⁻².s⁻¹) were treated with tungstate or L-NMMA under high light (1,600 µmol.m⁻².s⁻¹) for 2 days. These data was obtained from three independent replicates. Tungstate₁₀₀, Tungstate₂₀₀, 100, 200 µM Tungstate; L-NMMA₂₀₀, L-NMMA₃₀₀, 200, 300 µM L-NMMA; PTIO₂₀₀, 200 µM PTIO. * P< 0.05, ** P< 0.01 (t test).

**Figure 5.** NO was required for leaf cell death. Both 10-day-old wild type (WT) and noel seedlings cultivated under low light (400 µmol.m⁻².s⁻¹) were transferred to high light (1,600 µmol.m⁻².s⁻¹). Leaf symptoms and relative cell death (percentage of total leaf area) of wild type and noel seedlings induced by addition of 200 µM PTIO (an NO scavenger) and 2.5 mM SNP (an NO donor) for 2 DAT (days after treatment) (A and B) and 5 DAT (C and D) under high light. E, Effects of 200 µM PTIO and 2.5 mM SNP on relative cell death of wild type and noel suspension cell cultures. These data was obtained from three independent replicates.

**Figure 6.** Protein S-nitrosylation is involved in H₂O₂-induced leaf cell death. OsGSNOR relative expression level (noel set as 1.0) (A), GSNOR activity (B), SNO content (C), and S-nitrosylated proteins from biotin-switch assay immunoblotted with anti-biotin antibody (D) using 4-month-old wild type (WT), noel, and OsGSNOR noel transgenic lines grown under high light (1,600 µmol.m⁻².s⁻¹). E, Symptoms of
10-day-old wild type, noe1, and OsGSNOR noe1 transgenic lines after treatment with 200 µM PTIO, 2.5 mM SNP, and H2O2 (generated by 2.5 mM glucose plus 2.5 U/ml glucose oxidase system) for 3 days under low light (400 µmol.m⁻².s⁻¹). These data was obtained from three independent replicates. * P< 0.05, ** P< 0.01 (t test).

**Figure 7.** Identification of S-nitrosylated proteins from wild type (WT) plants and the noe1 mutant using LC/MS/MS. A, Number of S-nitrosylated proteins in wild type and noe1 plants. B, Functional categorization of S-nitrosylated proteins identified only from noe1 plants. Both 10-day-old wild type and noe1 seedlings cultivated under low light (400 µmol.m⁻².s⁻¹) were transferred to high light (1,600 µmol.m⁻².s⁻¹) for 2 days before sample collected.

**Figure 8.** A hypothetical model for the role of NO and SNOs in H2O2-induced leaf cell death in rice.
Table I. Redox related proteins were S-nitrosylated in noel plants.

| Proteins                             | RAP (OsID) | Hints to redox-related | Hints to cell death through S-nitrosylation A | Hints to cell death through other ways B |
|--------------------------------------|------------|------------------------|---------------------------------------------|-----------------------------------------|
| Glyceraldehyde-3-Phosphate Dehydrogenase | Os04g0545900 | Rodriguez-Pascual F et al. (2008) | Hara MR et al. (2005) | A |
| Thioredoxin                          | Os12g0188700 | Vieira et al. (2006) | Sumbayev et al. (2003) | A |
| Cytosolic Ascorbate Peroxidase       | Os07g0694700 | Andersen KM et al. (2009) | Vacca RA et al. (2004) | B |
| Glutaredoxin Subgroup II             | Os12g0175500 | Reynaert NL et al. (2006) | Saeed U et al. (2010) | B |
| Ferredoxin-Dependent Glutamate Synthase | Os07g0658400 | Garcia J et al. (2010) |                                  |                                      |
| Glutathione Reductase                | Os02g0613500 | Landino LM et al. (2004) |                                  |                                      |
| Monodehydroascorbate Reductase       | Os09g0567300 | Schweinzer E et al. (1995) |                                  |                                      |
Figure 1. Phenotypes of noe1 and map-based cloning of NOE1/OsCATC. SNO content (A) and phenotypes (B and C) of 3-month-old wild type (WT) and noe1 plants grown under high light (1600 µmol.m^-2.s^-1). D, Map-based cloning of NOE1/OsCATC. The NOE1/OsCATC gene was mapped in the interval of MT2 and MT8 on short arm of chromosome 3 and was delimited to a 61-kb region with 10 candidate genes between the MT19 and MT23 marker, the mutated sites of two distinct allelic mutant were shown in the gene LOC_Os03g03910. E, Expression patterns of OsCATA, OsCATB, and NOE1/OsCATC in different tissues analyzed by quantitative RT-PCR. F, Expression levels of OsCATA, OsCATB, and NOE1/OsCATC in wild type and noe1 leaves. These data was obtained from three independent replicates. * P< 0.05 (t test).
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Figure 8. A hypothetical model for the role of NO and SNOs in H₂O₂-induced leaf cell death in rice.