

The *Arabidopsis* **PARAQUAT RESISTANT2** gene encodes an S-nitrosogluthathione reductase that is a key regulator of cell death

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Metabolism of S-nitrosogluthathione (GSNO), a major biologically active nitric oxide (NO) species, is catalyzed by the evolutionally conserved GSNO reductase (GSNOR). Previous studies showed that the *Arabidopsis* *GSNOR1/HOT5* gene regulates salicylic acid signaling and thermotolerance by modulating the intracellular S-nitrosothiol level. Here, we report the characterization of the *Arabidopsis* *paraquat resistant2-1* (*par2-1*) mutant that shows an anti-cell death phenotype. The production of superoxide in *par2-1* is comparable to that of wild-type plants when treated by paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), suggesting that *PAR2* acts downstream of superoxide to regulate cell death. *PAR2*, identified by positional cloning, is shown to be identical to *GSNOR1/HOT5*. The *par2-1* mutant carries a missense mutation in a highly conserved glycine, which renders the mutant protein unstable. Compared to wild type, *par2-1* mutant has a higher NO level, as revealed by staining with 4,5-diaminofluorescein diacetate. Consistent with this result, wild-type plants treated with an NO donor display resistance to paraquat. Interestingly, the *GSNOR1/HOT5/PAR2* protein level, other than its steady-state mRNA level, is induced by paraquat, but is reduced by NO donors. Taken together, these results suggest that *GSNOR1/HOT5/PAR2* plays an important role in regulating cell death in plant cells through modulating intracellular NO level.

Keywords: *GSNOR1/HOT5/PAR2*, nitric oxide, paraquat, cell death, superoxide

Cell Research (2009) 19:1377–1387. doi: 10.1038/cr.2009.117; published online 6 October 2009

Introduction

In higher plants, programmed cell death (PCD) is an important mechanism to regulate multiple aspects of growth and development, as well as to remove damaged or infected cells during responses to environmental stresses and pathogen attacks [1–3]. PCD in plant cells shares many morphological similarities with that of animal cells, including organelle degeneration, nuclear condensation, nuclear DNA fragmentation and eventually

cell shrinkage. These common features suggest that a similar mechanism may be adopted by animal and plant cells to control PCD. However, no homologs corresponding to the major components of the animal PCD pathway have been found in any genomes of higher plants. Yet, little is known about the biochemical mechanism of PCD in plant cells. Most of our current knowledge on plant PCD has been obtained from studies of pathogen-plant interactions, in which the host often responds by eliciting a hypersensitive response (HR) around the initial infection site. HR has been characterized by a burst of reactive oxygen species (ROS), the accumulation of signaling molecules, such as nitric oxide (NO) and salicylic acid (SA), and the induction of *pathogenesis-related* genes, eventually followed by rapid cell death. HR acts to confine the pathogen and protect the plant, and is regulated by various signaling molecules, including ROS, NO and

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Received 23 June 2009; revised 29 June 2009; accepted 3 July 2009; published online 6 October 2009

SA [4, 5].

Genetic studies on the *Arabidopsis* lesion mimic mutants, which show an HR-syndrome in the absence of pathogen infection, have shed significant light on the molecular mechanism of plant PCD. Because of their spontaneous cell death phenotype and constitutive activation of the defense response, the lesion mimic mutants, represented by *accelerated cell death* [6] and *lesion stimulating disease resistance* mutants [7], could be affected in key regulatory loci controlling HR-type cell death. Using a nonpathogen approach by screening for mutants resistant to the PCD-inducing compound, fumonisin B₁ (FB₁), several *FB₁-resistant* (*fbr*) mutants have been identified [8, 9]. In contrast to that of the lesion mimic mutants, these *fbr* mutants show an anti-apoptotic phenotype [9–12]. Whereas *FBR6* and *FBR12* encode a plant-specific transcription factor, AtSPL14, and a eukaryotic translation initiation factor 5A, respectively [10, 12], *FBR11* encodes a subunit of serine palmitoyltransferase that catalyzes the first rate-limiting reaction of sphingolipid *de novo* synthesis [11, 13]. Although sphingolipid-regulated cell death has been attributed to ROS production [11, 14], the molecular link between these two classes of signaling molecules remains unclear.

In addition to FB₁, paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride), a nonselective herbicide, has also been shown as an efficient inducer of cell death in both animal and plant cells [15, 16]. In plant cells, paraquat mainly targets chloroplasts by accepting electrons from photosystem I and then reacting with oxygen to produce superoxide and hydrogen peroxide, which cause photooxidative stress [17, 18]. Consistently, chloroplast-targeted overexpression of superoxide dismutase (*SOD*) genes confers paraquat resistance in a number of transgenic species [19–21]. Interestingly, the *Arabidopsis* radical-induced cell death1 (*rcd1*) mutant displays resistance to paraquat, and this phenotype is associated with the increased expression of plastidic Cu/Zn *SOD* and ascorbate peroxidase (*APX*) genes [22]. These results suggest that paraquat-induced cell death is mediated by a ROS-based signaling pathway.

NO has been considered as an important signaling molecule involved in both biotic and abiotic stress responses, as well as in many aspects of plant growth and development [23–26]. The balance between intracellular NO and ROS levels has been shown to be a key determinant for HR [26–30]. The ROS and NO levels appear to be reciprocally controlled through, at least partly, related scavenging enzymes, and NO has also been proposed to function as an antioxidant under certain conditions [28, 29, 31–33]. Moreover, *S*-nitrosylation, the covalent addition of an NO molecule to a cysteine thiol of a protein

or a peptide, is a key destination of the gaseous hormone and a redox-based posttranslational modification mechanism. *S*-nitrosylation appears to be an important mechanism to regulate the activity and stability of many proteins and enzymes [24, 34–38]. In this complicated, yet not well-understood process, *S*-nitrosoglutathione (GSNO), derived by *S*-nitrosylation of the antioxidant tripeptide glutathione, is a major reservoir of biologically active NO. The evolutionally conserved GSNO reductase (GSNOR) is a key enzyme catalyzing the reduction of GSNO, thus controlling intracellular levels of GSNO and *S*-nitrosothiols (SNOs) [39, 40].

In *Arabidopsis*, the single-copied *GSNOR1* gene has been genetically and biochemically characterized [41–47]. Recombinant *Arabidopsis* GSNOR1 protein shows enzymatic activity of reducing GSNO *in vitro* [45]. Moreover, mutations in *GSNOR1* cause developmental defects and altered responses to both biotic and abiotic stresses [42, 46]. A loss-of-function mutation in the *GSNOR1* gene (*gsnor1-3*) causes an increased SNO level, accompanied with compromised defense responses mediated by distinctive resistance genes, as well as both basal and nonhost resistances [42]. Conversely, gain-of-function mutations in *GSNOR1* result in reduced SNO formation [42]. These results indicate that GSNOR1 is a positive regulator of SA signaling. More recently, the *Arabidopsis* sensitive to hot temperatures5 (*HOT5*) gene was characterized to be identical to *GSNOR1* [46]. Several allelic *gsnor1/hot5* mutants show a similar phenotype characteristic of compromised heat acclimation and elevated levels of NO species, illustrating an important role of SNO homeostasis in the regulation of abiotic stress response as well [46]. Apart from its role in SA signaling and the abiotic stress response, it however remains unknown if the intracellular NO level modulated by *GSNOR1/HOT5* is involved in any other cellular activities. In this study, we report the identification and characterization of an anti-cell death mutant *paraquat resistant2-1* (*par2-1*) that is allelic to *gsnor1/hot5*. Our results suggest that *GSNOR1/HOT5/PAR2* plays an important role in the regulation of cell death.

Results

Genetic screen and analysis of *par* mutants

When germinated in the presence of paraquat, growth and development of wild-type *Arabidopsis* plants were strongly inhibited. Cotyledons remained small and brown-yellowish and occasionally turned green-yellowish. Root growth and true leaf initiation were arrested, and the plants died eventually (Supplementary information, Figure S1A and S1B). Accompanying cell

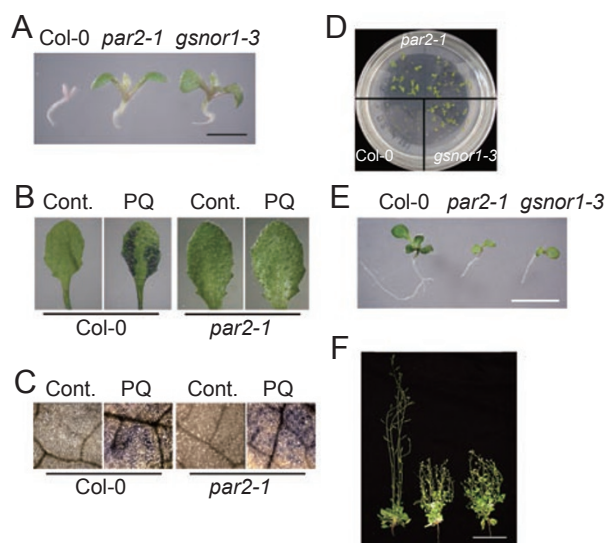


Figure 1 The *par2-1* mutant phenotype. **(A)** Wild type (WT; Col-0), *par2-1* and *gsnor1-3* seedlings (7-day-old) germinated and grown in the presence of 2 μ M paraquat. Bar, 1 mm. **(B)** Paraquat-induced cell death. Col-0 and *par2-1* seedlings (4-week-old) were treated with (PQ) or without (Cont.) 5 μ M paraquat for 24 h by spraying. Leaves were then detached from treated plants and stained with Evans blue. Under the assay conditions, less than 10% of untreated wild-type leaves and *par2-1* leaves (treated or untreated) were stained as positive for cell death, whereas more than 90% paraquat-treated wild type leaves were stained as positive. **(C)** Accumulation of superoxide induced by paraquat. Wild type and *par2-1* seedlings (4-week-old) treated as described in **(B)** for 6 h, and leaves were collected and then stained with nitroblue tetrazolium (NBT). Superoxide accumulation was shown as blue precipitates. Approximately 10%-20% of untreated leaves could be stained by NBT at varying degrees, and 80%-90% of paraquat-treated leaves were stained as positive by NBT. **(D)** Col-0, *par2-1* and *gsnor1-3* seedlings (2-week-old) germinated and grown in the presence of 0.8 μ M FB₁. **(E)** Wild type, *par2-1* and *gsnor1-3* seedlings (10-day-old) germinated and grown on MS medium. Bar, 1 mm. **(F)** Wild type, *gsnor1-3* and *par2-1* (from left to right) plants (6-week-old) grown in soil. Bar, 5 cm.

death, paraquat induced the production of superoxide and hydrogen peroxide in wild-type leaves (Supplementary information, Figure S1D and S1E). When treated with paraquat, detached leaves rapidly turned bleached under light, but remained green when cultured in the dark (Supplementary information, Figure S1C), consistent with the photooxidative stress effect of the herbicide. We reasoned that mutations that render plants resistant to paraquat may represent important genetic loci that are involved in either detoxification or the regulation of cell death in plant cells. Therefore, we carried out a genetic screen for *Arabidopsis par* mutants by surveying ethyl-

methanesulphonate (EMS)-mutagenized seeds (in Columbia-0 or Col-0 and Wassilewskija or WS background) on MS medium [48] containing 2 μ M paraquat. From this screen, we have recovered 12 *par* mutants, which were in the Col-0 background (2 mutants) and the WS background (10 mutants). These *par* mutants showed resistance to paraquat at varying degrees. Here, we present a detailed study on a representative mutant, *par2-1*.

The *par2-1* mutant was in the Col-0 background. We crossed *par2-1* with wild-type Col-0 plants, and the paraquat-resistant phenotype was scored in F1 and F2 populations. All tested F1 plants (25 plants) were sensitive to paraquat. Among the analyzed F2 plants (derived from self-pollinated F1 plants), the paraquat-resistant phenotype was segregated in a 1:3 ratio (resistant:sensitive = 54:164; $\chi^2 = 0.90$), indicating that the mutation is recessive in a single nuclear gene. F1 plants obtained from crosses between *par2-1* and other *par* mutants showed a wild-type phenotype, indicating that *par2-1* was not allelic to any other *par* mutants identified in this study. The original *par2-1* mutant was backcrossed with Col-0 twice, and homozygous progenies of F3 or subsequent generations were used in all experiments described below.

The *par2-1* mutant phenotype

When germinated and grown in the presence of paraquat, *par2-1* showed resistance to paraquat, whereas growth and development of wild-type plants were completely inhibited (Figure 1A). In contrast to that of wild-type leaves, paraquat-induced cell death was barely detected in *par2-1* leaves (Figure 1B), suggesting that paraquat-induced cell death program is impaired in the mutant. Because paraquat has been known to induce the generation of ROS, we examined the accumulation of superoxide and hydrogen peroxide in *par2-1* leaves treated with paraquat. When treated with paraquat, the production of superoxide and hydrogen peroxide in *par2-1* was comparable to that in wild-type leaves (Figure 1C and data not shown). This result indicates that the paraquat-induced production of superoxide does not require *PAR2*, which likely acts downstream of ROS to regulate cell death. In agreement with this notion, *par2-1* also showed resistance to FB₁ (Figure 1D), a fungal toxin that can efficiently induce PCD in plants, likely via regulating ROS generation [8, 9, 11, 14].

To ask if the paraquat-induced cell death involves nuclear DNA fragmentation, a hallmark of PCD, we analyzed paraquat-treated wild type and mutant protoplasts by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Positive TUNEL signals were easily detected in paraquat-treated protoplasts, but rarely ob-

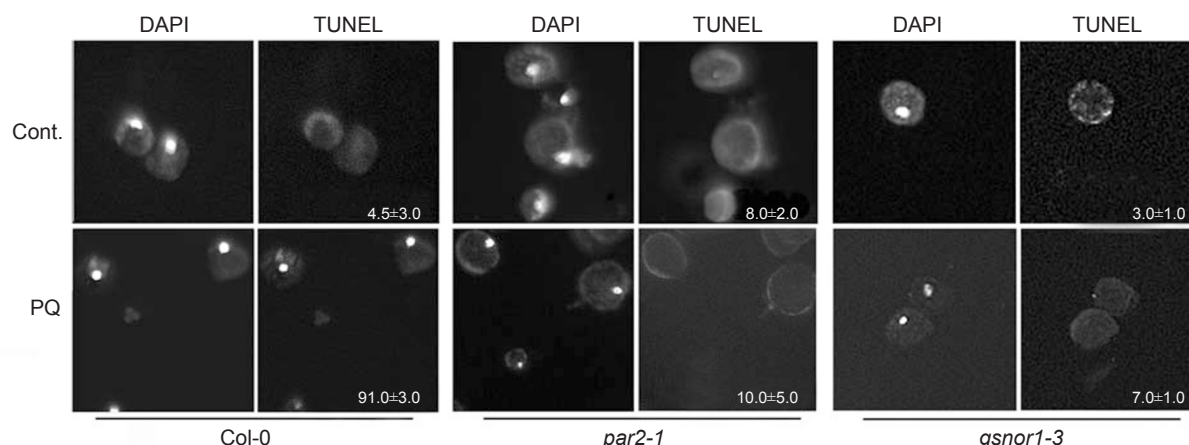


Figure 2 Nuclear DNA fragmentation induced by paraquat. Protoplasts prepared from leaves derived from 4-week-old wild-type seedlings were treated with (PQ) or without (Cont.) 0.5 μ M paraquat for 6 h, and then subjected to terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis of free 3'-OH group of DNA. Nuclear DNA was stained with 4',6-diamino-2-phenylindole (DAPI). Numbers inside the TUNEL panels represent percentage of TUNEL-positive protoplasts and standard derivations. The data were mean values obtained from three independent experiments.

served in water-treated wild-type protoplasts. However, substantially reduced TUNEL signals were detected in paraquat-treated protoplasts derived from *par2-1* leaves compared to that of wild-type plants (Figure 2). Consistent with its light-dependent killing effect, paraquat treatment in the dark did not induce apparent TUNEL-positive signals in protoplasts derived from either wild type or *par2-1* leaves (data not shown). Taken together, these results suggest that the *par2-1* mutant phenotype involves an anti-cell-death mechanism related to the inhibition of PCD.

Under normal growth conditions, *par2-1* displayed a semi-dwarf and bushy phenotype with reduced fertility (Figure 1E and 1F), suggesting that *PAR2* is also required for normal growth and development.

Molecular cloning of *PAR2*

Using an F2 population obtained from crosses of *par2-1* \times Landsberg *erecta* (*Ler*), we mapped the mutation onto chromosomes V, between an SSLP marker CIW9 and an InDel marker K9E15. By monitoring genetic recombination in a population of 3363 F2 mutant plants, we located *PAR2* between markers F6B6 and MRH10-5, an ~47-kb region containing 11 open reading frames (Figure 3A). DNA sequencing analysis of all 11 genes revealed a mutation in At5g43940, characterized as a G-to-A transition in exon 6, which converts a glycine into an aspartic acid at residue 224 (Figure 3A). This glycine residue is highly conserved in related proteins across different kingdoms (Figure 3B).

To verify the identity of the candidate *PAR2* gene, we

performed a genetic complementation experiment. A 3.3-kb wild type genomic DNA fragment, which contains the putative promoter region, 5'-untranslated region (UTR), coding sequence and 3'-UTR of At5g43940, was cloned into a binary vector. The resulting construct was transformed into *par2-1* mutant plants by floral dipping. Among the analyzed eight independent transgenic lines, all plants showed a phenotype similar to that of wild type, including the restored sensitivity to paraquat and normal growth and development (Figure 3C and data not shown), suggesting that the observed *par2-1* mutant phenotype was caused by a mutation in At5g43940.

In previous studies, At5g43940 has been characterized to encode a GSNOR [41, 42, 44, 45]. A loss-of-function mutation in *GSNOR1* (*gsnor1-3*) caused no *GSNOR1* transcript accumulation [42], thus likely representing a null mutation (see Figure 3A). The *gsnor1-3* mutant showed a phenotype similar to *par2-1*, including the paraquat resistance and abnormal growth and development (Figures 3D and 1E-1F). To test possible allelism between *par2-1* and *gsnor1-3*, we crossed these two mutants and analyzed the resulting F1 plants. When germinated in the presence of paraquat, all tested F1 plants were resistant to the herbicide and showed a dwarf and bushy phenotype similar to their parents (Figure 3D and data not shown). Moreover, the At5g43940 transgene also fully restored the sensitivity of *gsnor1-3* to paraquat and rescued the developmental defects (Figure 3E and data not shown). Therefore, *par2-1* and *gsnor1-3* are allelic, and At5g43940 represents the *PAR2* gene. Five independent mutant alleles (*hot5* alleles) of *GSNOR1* have

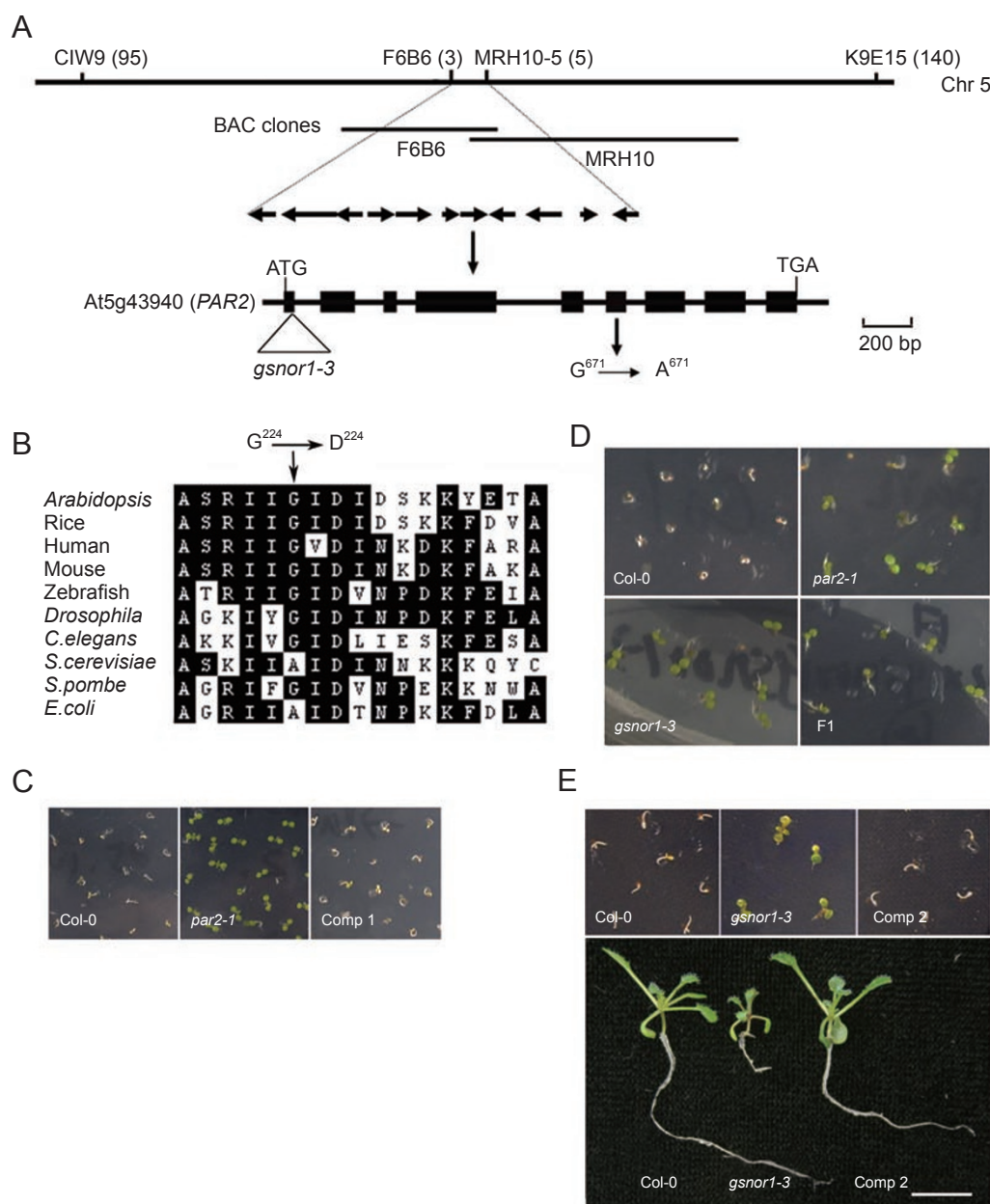


Figure 3 Molecular characterization of the *PAR2* gene. **(A)** A schematic map of positional cloning of *PAR2*. Top shows region with major markers used in genetic mapping of *PAR2*. Numbers in parentheses indicate recombinants found in the mapped population (3 363 F2 mutant plants) of a given marker. Middle: annotated genes between two markers F6B6 and MRH10-5. Arrows denote the transcribed direction of each gene. Bottom: structure of *PAR2*. Exons and introns are represented by filled boxes and solid lines, respectively. The position and nature of the mutated nucleotide are shown. The position of the T-DNA insertion in *gsnor1-3* [42] is also shown. **(B)** Amino acid residue sequences of GSNOR1/HOT5/*PAR2* and its homologs from different species. The alignment is around the mutated residue (Gly²²⁴). Conserved residues are shaded. Accession numbers: *Arabidopsis*: NP_199207; *Rice*: BAD21999; *Human*: NP_000662; *Mouse*: NP_031436; *Zebrafish*: NP_571924; *Drosophila*: NP_524310; *Caenorhabditis elegans*: NP_741507; *Saccharomyces cerevisiae*: NP_010113; *Saccharomyces pombe*: NP_588247; *Escherichia coli*: NP_414890. **(C)** Molecular complementation of the *par2-1* mutant. Seven-day-old seedlings were germinated and grown on MS medium containing 1 μ M paraquat. Comp 1: *par2-1* plants carrying a pER10-*PAR2* transgene (see text). **(D)** Allelism test of *par2-1* and *gsnor1-3* mutants. Five-day-old seedlings were germinated and grown on MS medium containing 1 μ M paraquat. F1: F1 progenies obtained from a cross between *par2-1* and *gsnor1-3*. **(E)** Molecular complementation of the *gsnor1-3* mutant. Top: 10-day-old seedlings germinated and grown on MS medium containing 1 μ M paraquat. Bottom: 2-week-old seedlings germinated and grown on MS medium. Comp 2: *gsnor1-3* plants carrying a pER10-*PAR2* transgene. Bar, 1 cm.

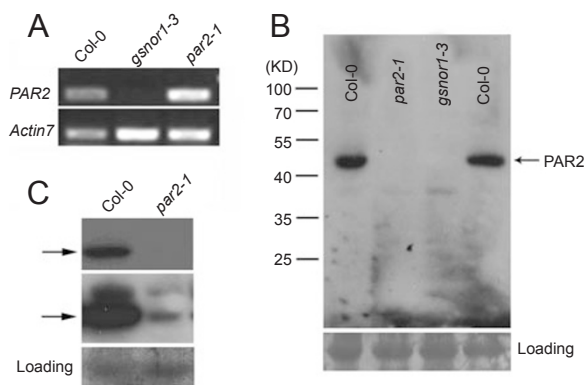


Figure 4 Expression of *GSNOR1/HOT5/PAR2* in *par2-1* and *gsnor1-3*. **(A)** Expression of *GSNOR1/HOT5/PAR2* in wild type, *par2-1* and *gsnor1-3* plants (4-week-old seedlings) analyzed by reverse transcription (RT)-PCR. *Actin7* was used as an internal control. **(B)** Western blot analysis of *GSNOR1/HOT5/PAR2* protein in wild type, *par2-1* and *gsnor1-3* plants (4-week-old seedlings). Positions of molecular weight markers (in Kilo Daltons or KD) are shown at the left of the panel. Each lane contains 30 μ g total proteins. The loading control was determined by staining the blot with Ponceau S. **(C)** Western blot analysis of *GSNOR1/HOT5/PAR2* protein in wild type and *par2-1* plants (4-week-old seedlings). *GSNOR1/HOT5/PAR2* protein could be occasionally detected in *par2-1*. Wild type and mutant *PAR2* proteins are denoted by arrows. Short- (top) and long-exposure (middle) of the same blot are shown.

been recently characterized, which show compromised acclimation to heat stress as well as various developmental abnormalities similar to those observed in *par2-1* [46].

We obtained a *GSNOR1/HOT5/PAR2* cDNA clone by reverse transcription (RT)-PCR, which was identical to an EST clone (accession number: AK226412). Comparison of the genomic and cDNA sequences revealed that *GSNOR1/HOT5/PAR2* contained 9 exons, encoding a protein of 379 amino acid residues (Figure 3A). We noticed that *GSNOR1/HOT5/PAR2* was previously reported to contain 8 exons, although the same size of the protein (379 amino acid residues) was reported [46]. This discrepancy might be due to variants of alternative splicing or incorrect annotation.

PAR2 encodes an *GSNOR*

GSNOR is a highly conserved protein found in all living organisms from bacteria to higher eukaryotes, and catalyzes the metabolism of GSNO to form GSSG and NH_3 as main products [40, 49]. *GSNOR1* is a single-copied gene in *Arabidopsis*, and has been characterized in some detail [41-46]. Loss- or gain-of-function mutations in *GSNOR1* caused altered innate immunity against a variety of pathogens and altered intracellular SNO levels

[42, 43]. Moreover, *GSNOR1/HOT5* also plays a critical role in thermotolerance [46].

Similar to the results obtained from a previous study [42], expression of *GSNOR1/HOT5/PAR2* was not detectable in *gsnor1-3* by either RT-PCR or northern blot analysis (Figure 4A and data not shown). In *par2-1*, expression of *GSNOR1/HOT5/PAR2* was comparable to that in wild type (Figure 4A), indicating that the muta-

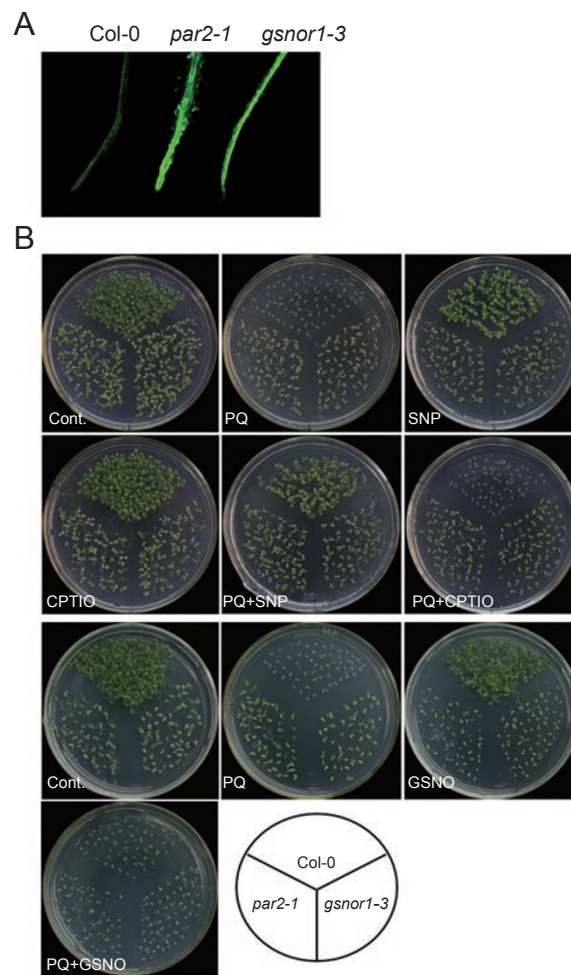


Figure 5 Increased NO levels are correlated to the paraquat resistance. **(A)** Increased NO contents in *par2-1* and *gsnor1-3* roots. Two-week-old seedlings germinated and grown on MS medium were stained with DAF-2DA. Roots were observed and photographed under a fluorescent microscope. **(B)** Wild type (Col-0), *par2-1* and *gsnor1-3* seedlings germinated and grown under different conditions as indicated in the panel. Top two rows: 10-day-old seedlings; Bottom two rows: 12-day-old seedlings. Genotypes of the tested samples are indicated at the bottom. Cont.: control (MS medium); PQ: paraquat. Concentrations of reagents used in the experiment: paraquat, 1 μ M; SNP, 100 μ M; CPTIO, 150 μ M; GSNO, 500 μ M.

tion did not affect transcription or stability of the mutant mRNA. However, the mutant protein was remarkably reduced as revealed by an anti-PAR2 polyclonal antibody (Figure 4B and 4C), suggesting that the *par2-1* mutation renders the mutant protein unstable and that the highly conserved Gly²²⁴ plays an important role in regulating the stability of PAR2 protein.

Paraquat resistance is enhanced by NO donor SNP

Previous studies indicated that *gsnor1-3* and *hot5* mutants had higher levels of SNO and NO species than wild-type plants [42, 43, 46]. To analyze the intracellular NO level in *par2-1*, we stained wild type and mutant seedlings with 4,5-diaminofluorescein diacetate (DAF-2DA), a fluorescent dye for *in vivo* imaging of NO [50]. In wild-type roots, specific fluorescent signals were slightly detectable under the assay condition. However, more intense signals were observed in *par2-1* and *gsnor1-3* mutant roots (Figure 5A), consistent with the observation made with *hot5* mutant alleles [46].

Because NO has been reported to act as an antioxidant [28, 32, 33], we reasoned that the paraquat-resistance phenotype of *par2-1* may be related to the increased intracellular NO level in the mutant. To test this possibility, wild type, *par2-1* and *gsnor1-3* were germinated and grown in the presence of NO donors, sodium nitroprusside (SNP) and GSNO, or the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy-3-oxide (CPTIO). When germinated and grown in the presence of SNP, whereas *par2-1* and *gsnor1-3* showed stronger resistance to paraquat, wild-type plants also displayed resistance to the herbicide (Figure 5B). Treatment with GSNO or CPTIO did not apparently alter sensitivity to paraquat (Figure 5B). A previous study found that CPTIO, applied alone or combined with SNP, caused apparent phenotype in *hot5-1* seedlings in a heat-inhibited hypocotyl elongation assay. However, CPTIO did not have effects on wild-type seedlings, although SNP caused remarkable heat-sensitivity phenotype in the same assay [46]. Presumably, heat-inhibition of hypocotyl elongation is a more sensitive assay than paraquat resistance. These results suggest that SNP is capable of inducing paraquat resistance in both wild-type and mutant plants.

Accumulation of GSNOR1/HOT5/PAR2 protein is regulated by paraquat and NO donors

Because *GSNOR1/HOT5/PAR2* directly modulates the NO level, which, in turn, correlates with resistance to paraquat that induces ROS generation, it is likely that expression of *GSNOR1/HOT5/PAR2* is subjected to the regulation by oxidative stresses and the NO level. To ex-

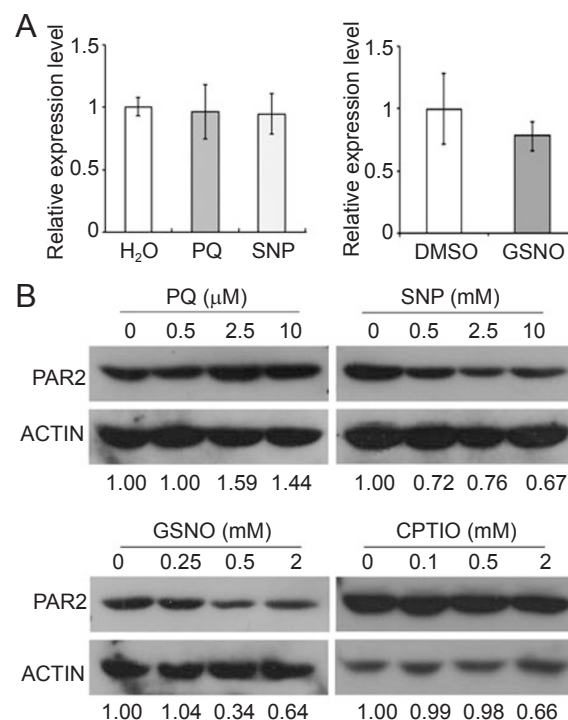


Figure 6 The accumulation of GSNOR1/HOT5/PAR2 protein is regulated by paraquat and NO donors. **(A)** Expression of *GSNOR1/HOT5/PAR2* analyzed by qRT-PCR. Wild type (Col-0) seedlings (2-week-old) germinated and grown on MS medium were treated with paraquat (1 μM), SNP (200 μM) or GSNO (400 μM) for 12 h, and then used for the preparation of total RNA. Water- or DMSO (dimethyl sulfoxide, a solvent for GSNO)-treated seedlings were used as controls. The relative expression level of *GSNOR1/HOT5/PAR2* was determined using *Actin7* as an internal control. Data presented are mean values of two independent experiments (biological repeats) with standard deviations (bars in the graph). **(B)** Effects of paraquat, SNP, GSNO and CPTIO on the accumulation of GSNOR1/HOT5/PAR2 protein analyzed by western blot. Wild type seedlings (2-week-old) were treated with different concentrations of compounds (indicated on the top of the panel) for 12 h. Protein extracts were prepared and then subjected to western blot analysis using an anti-PAR2 antibody. Each lane contains 30 μg total proteins. The blot was striped and reanalyzed by an anti-actin antibody. Relative level of PAR2 protein was normalized with the actin level and indicated at the bottom of the blots.

plore this possibility, we analyzed *GSNOR1/HOT5/PAR2* expression by quantitative RT-PCR (qRT-PCR) under different conditions. The steady-state level of *GSNOR1/HOT5/PAR2* mRNA did not appear to be altered by a variety of treatments, including paraquat, SNP and GSNO (Figure 6A). We next examined the *GSNOR1/HOT5/PAR2* protein level by western blot. We observed that paraquat treatment caused an increased accumulation of

GSNOR1/HOT5/PAR2 protein (Figure 6B). Conversely, the GSNOR1/HOT5/PAR2 protein level was reduced when treated by NO donors, SNP and GSNO (Figure 6B). The GSNOR1/HOT5/PAR2 protein level did not appear to be significantly altered when treated with CPTIO alone or in combination with GSNO (Figure 6B and data not shown). These results indicate that the accumulation of GSNOR1/HOT5/PAR2 protein is enhanced by paraquat, but is negatively regulated by NO donors, suggesting possible reciprocal regulation on the accumulation of GSNOR1/HOT5/PAR2 protein by ROS and NO.

Discussion

In this study, we show that paraquat-induced cell death is light dependent and shares characteristics of PCD by the induction of superoxide and nuclear DNA fragmentation. Moreover, we have identified and characterized the *par2-1* mutant, revealing an important function of *GSNOR1/HOT5/PAR2* in the regulation of ROS-mediated cell death. The *par2-1* mutant was identified by its resistance to paraquat, which has been known to induce the generation of ROS and free radicals [15, 17, 18]. It has been estimated that paraquat treatment on light-grown ryegrass and duckweed produces excessive amount of hydroxyl radicals equivalent to that produced by 10 000 rads of γ irradiation, a dose that is able to induce rapid cell death in plants [17]. Thus, the killing effect of paraquat has been mainly attributed to the production of excessive ROS [15, 17, 18]. Accordingly, resistance to paraquat in plants can be ascribed to two distinctive mechanisms. One possibility is the detoxifying effect such as by the removal of excessive amount of ROS and reactive hydroxyl radicals. The *Arabidopsis rcd1* mutant, for example, displays resistance to paraquat, and this phenotype is associated with the increased expression of plastidic *SOD* and *APX* genes [22]. Similarly, the paraquat-induced cell death can also be suppressed by overexpression of a chloroplast-targeted *SOD* gene [20] or a thylakoidal *APX* gene [51]. When treated with paraquat, the *par2-1* mutant displays reduced cell death, but accumulates superoxide at a level similar to that in wild type, suggesting that *GSNOR1/HOT5/PAR2* may not be directly involved in the regulation of superoxide production or turnover.

A second possibility is that *GSNOR1/HOT5/PAR2* acts downstream of superoxide to regulate a cell-death signaling pathway, and the *par2-1* mutation blocks this signaling pathway that is activated by paraquat-induced superoxide or free radicals. Alternatively, *GSNOR1/HOT5/PAR2* may regulate a superoxide-independent cell-death signaling pathway. Because a major physiological

effect of paraquat is the induction of superoxide and free radicals in plant cells, it is reasonable to assume that *PAR2* functions downstream of superoxide to regulate cell death. This view is supported by the observation that *par2-1* displays resistance to FB₁, which also induces the formation of ROS [8, 9, 11].

The superoxide-insensitivity phenotype of *gsnor1/hot5/par2* mutants suggests that the wild-type allele is a component involved in a superoxide-mediated cell-death signaling pathway, and that the anti-cell death phenotype results from blocked superoxide signaling. What is the possible mechanism of the reduced superoxide-sensitivity of *par2-1*? *PAR2* encodes a GSNOR involved in the regulation of a wide range of biotic and abiotic stress responses as well as plant growth and development. Consistent with the biochemical nature of GSNOR, multiple *gsnor1/hot5/par2* mutant alleles show an increased NO level, whereas overexpression of *GSNOR1/HOT5/PAR2* causes a reduced NO level [42, 43, 46]. These results imply that the altered NO level may suppress cell death induced by paraquat in *par2-1*. Consistent with this notion, properly balanced homeostasis of ROS and NO has been found to play a critical role in the regulation of HR cell death [27-29]. Although the underpinning mechanism of the ROS-NO interaction remains elusive, NO donors have been demonstrated to regulate ROS levels by inhibiting two antioxidant enzymes, catalase and *APX*, in tobacco [31]. Moreover, reciprocal scavenging of NO and O₂⁻ was shown to cause the formation of peroxynitrite anion ONOO⁻, a compound that is less toxic in plants [28]. Likewise, a similar mechanism may be operating in *par2-1*, and the NO donor SNP confers paraquat resistance, presumably by exerting an antagonistic effect against the oxidative stress through increasing the NO level. This view is supported by the observation that SNP also confers paraquat resistance to potato leaves [52], and acts as an antioxidant to delay PCD induced by gibberellin in barley aleurone layers [33]. Again, consistent with the reciprocal regulation of NO and ROS, the accumulation of GSNOR1/HOT5/PAR2 protein is induced by paraquat, but is reduced when treated by SNP and GSNO. This result may partly explain the paraquat-resistance phenotype of *par2-1* and SNP-treated wild-type plants, both of which have reduced GSNOR1/HOT5/PAR2 protein levels, which may act as a key regulator of the NO-ROS interaction to regulate cell death in plants.

Finally, GSNOR1/HOT5/PAR2 may also regulate plant cell death through *S*-nitrosylation. In animal cells, the regulatory role of *S*-nitrosylation in apoptosis has been well appreciated [53-55]. In *Arabidopsis*, a number of *S*-nitrosylated proteins have been identified under different conditions using proteomic approaches [56-

58]. In particular, *S*-nitrosylation of peroxiredoxin II E has been demonstrated to inhibit its activity involved in hydroperoxide reduction and peroxynitrite detoxification [37], and *S*-nitrosylation of NPR1 is critical for the regulation of redox-based conformational changes that directly determine the NPR1 activity [35]. In addition, the *Arabidopsis* METACASPASE9 activity is regulated by *S*-nitrosylation, providing a possible link between the redox-based posttranslational modification mechanism and biochemical execution of cell death in plant cells [38]. Among these proteins, whereas the level of biologically active monomeric NPR1 is reduced in *gsnor1-3*, which is presumably caused by an altered *S*-nitrosylation pattern [35], it is unclear whether *S*-nitrosylation of other proteins and additional unidentified targets plays a regulatory role in PCD. It will be challenging and of great interest to clarify these questions, which should shed new light on the molecular mechanism of plant PCD.

Materials and Methods

Plant materials, growth conditions and genetic screen for par mutants

The Col-0, WS and *Ler* accessions of wild-type *Arabidopsis* were used in this study. Seeds of *gsnor1-3* [42] were kindly provided by Dr Gary Loake. Plants were grown under a 16 h light/8 h dark cycle or continuous white light ($120\text{--}130\ \mu\text{mol m}^{-2}\text{ s}^{-1}$) at 22 °C in soil or on an MS medium [48] containing 3% sucrose and 0.8% agar.

To screen *par* mutants, EMS-mutagenized M2 seeds, ~15 000 seeds in the Col-0 background and 45 000 seeds in the WS background, were germinated and grown on MS agar plates containing 2 μM paraquat (Sigma, Hong Kong, China) for 7–10 days. Upon germination, whereas majority of seedlings were bleached or yellowish, putative *par* mutants became green or light green. These putative *par* mutant seedlings were transferred onto a fresh MS medium without paraquat and then to soil until maturation. M3 seeds of the putative *par* mutants were rescreened under the same conditions, and 12 mutants (2 in Col-0 and 10 in WS background) were recovered. Genetic and allelism analyses of the *par* mutants were performed by pair-wise crossing of individual mutants, followed by assessing phenotypes and genetic segregation patterns in F1 and F2 generations. In all cases, reciprocal crosses were performed and similar results were obtained.

Genetic mapping of par2-1

F2 seeds derived from crosses between *par2-1* (Col-0) and wild-type *Ler* were germinated and grown on MS medium containing 2 μM paraquat for 10–15 days, and paraquat-resistant progenies were selected for genetic mapping. The *par2-1* mutation was roughly mapped onto chromosome V, between CIW9 and K9E15 by bulk segregate analysis. An F2 population of 3 363 mutant plants was then analyzed to define the *par2-1* mutation between F6B6 and MRH10-5 (see Supplementary information, Table S1 for sequences of all primers), two markers spanning ~47 kb. PAR2 candidate gene was deduced by DNA sequencing analysis of all annotated genes in this region.

Analysis of cell death, superoxide, hydrogen peroxide and nuclear DNA fragmentation

Detection of cell death, superoxide and hydrogen peroxide was carried out by staining leaves with Evans blue, nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine, respectively, essentially as previously described [11]. All experiments were repeated for 3–5 times (biological repeats), and 10–15 rosette leaves collected from multiple seedlings (4–5-week-old) were analyzed in each experiment.

Preparation of protoplasts and analysis of nuclear DNA fragmentation in protoplasts were performed as previously described [12] with minor modifications. Protoplasts were incubated with or without 0.5 μM paraquat for 6 h at 22 °C under continuous white light, fixed overnight in 2% paraformaldehyde and then subjected to TUNEL analysis, as previously described [12]. Nuclear DNA was stained with 4',6-diamino-2-phenylindole.

In vivo imaging of NO

Analysis of NO contents in *Arabidopsis* roots were performed by DAF-2DA staining as described [59] with minor modifications. Briefly, 2–3-week-old seedlings were pre-incubated for 2 h at room temperature in a solution of 0.1 mM CaCl_2 , 10 mM KCl, 10 mM MES-Tris, pH 5, and then stained with 10 μM DAF-2DA for 45 min. After brief rinse with water, the sample was observed and photographed under a fluorescent microscope equipped with a CCD camera (Olympus BX51).

Preparation of anti-PAR2 antibodies and western blot analysis

A *GSNOR1/HOT5/PAR2* cDNA fragment (nucleotides 460–951), encoding amino acid residues 154–317 of the protein, was cloned into the *Bam*HI and *Sal*I sites of a pET-28a vector (Novagen) to express a 6×His-PAR2 recombinant protein. The purified recombinant protein was used to immunize rabbits to generate the antiserum. The anti-PAR2 antiserum was extensively characterized using protein extracts prepared from wild type, *par2-1* and *gsnor1-3* plants as well as purified recombinant GSNOR1/HOT5/PAR2 protein, and then was directly used in all experiments in this study.

Protein extracts were prepared by grinding plant materials in liquid nitrogen and immediately mixing with grinding buffer (50 mM Tris, pH7.5, 150 mM NaCl, 10 mM MgCl_2 , 0.1% Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride). After centrifugation twice at 4 °C for 10 min in a microcentrifuge at full speed, the supernatant was subjected to SDS-PAGE. The protein concentration was determined by the Bradford method using BSA as a standard [60]. Typically, 30–60 μg of total proteins were used for SDS-PAGE. After the run, the proteins were electrically transferred onto a PVDF membrane (Whatman), and then detected with the anti-PAR2 antiserum as a primary antibody (~5 000-fold dilution). After incubation with a secondary antibody (HRP-conjugated goat anti-rabbit IgG; Pierce Biotechnology), the signal was detected using a SuperSignal Western Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology) according to the manufacturers instructions.

Molecular manipulations

All molecular manipulations were performed according to standard methods [61]. A *GSNOR1/HOT5/PAR2* (At5g43940) genomic DNA fragment of 3.3 kb was obtained by PCR from wild-type

(Col-0) plants, verified by restriction digests and DNA sequencing, and then cloned into the *Xho*I and *Spe*I sites of a binary vector pER10 to yield pER10-*PAR2*. This genomic fragment contained a promoter sequence of 0.8 kb upstream from the putative translation start codon, starting from the 3'-UTR of At5g43935, a gene in a head-to-tail configuration with *GSNOR1/HOT5/PAR2* (At5g43940). The pER10-*PAR2* was transformed into both *par2-1* and *gsnor1-3* mutant plants by the floral dip method [62].

Total RNA was prepared using the TRizol reagents (Invitrogen) following the manufacture's instructions. Northern blot, RT-PCR and real-time qRT-PCR were carried out as previously described [12].

Acknowledgments

We thank Dr Gary Loake (University of Edinburgh, UK) for providing *gsnor1-3* seeds. We are grateful to Drs Chuanyou Li, Shuhua Yang and Yiqin Wang for critically reading the manuscript. This study was supported by grants from the National Natural Science Foundation of China (30330360), the Ministry of Science and Technology of China (2006AA10A112) and the Chinese Academy of Sciences (KSCX2-YW-N-015).

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