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The *Arabidopsis* Prohibitin Gene *PHB3* Functions in Nitric Oxide–Mediated Responses and in Hydrogen Peroxide–Induced Nitric Oxide Accumulation [C] [W]

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To discover genes involved in nitric oxide (NO) metabolism, a genetic screen was employed to identify mutants defective in NO accumulation after treatment with the physiological inducer hydrogen peroxide. In wild-type *Arabidopsis thaliana* plants, NO levels increase eightfold in roots after H_2O_2 treatment for 30 min. A mutant defective in H_2O_2-induced NO accumulation was identified, and the corresponding mutation was mapped to the prohibitin gene *PHB3*, converting the highly conserved Gly-37 to an Asp in the protein's SPFH domain. This point mutant and a T-DNA insertion mutant were examined for other NO-related phenotypes. Both mutants were defective in abscisic acid–induced NO accumulation and stomatal closure and in auxin-induced lateral root formation. Both mutants were less sensitive to salt stress, showing no increase in NO accumulation and less inhibition of primary root growth in response to NaCl treatment. In addition, light-induced NO accumulation was dramatically reduced in cotyledons. We found no evidence for impaired H_2O_2 metabolism or signaling in the mutants as H_2O_2 levels and H_2O_2-induced gene expression were unaffected by the mutations. These findings identify a component of the NO homeostasis system in plants and expand the function of prohibitin genes to include regulation of NO accumulation and NO-mediated responses.

INTRODUCTION

Nitric oxide (NO) is a reactive nitrogen species that acts as an intermediate in multiple signaling pathways in plants. These pathways control a diverse set of processes, including programmed cell death, stomatal movements, auxin-induced lateral root formation, abiotic stress, and defense responses (reviewed in Lamattina et al., 2003; Neill et al., 2003; Wendehenne et al., 2004; Delledonne, 2005; Besson-Bard et al., 2008; Palavan-Unsal and Arisan, 2009; Yoshioka et al., 2009). Many environmental and hormonal stimuli, such as anoxia, abscisic acid (ABA), light, salt stress, pathogens, and elicitors, induce a rapid increase in NO levels. Treating plants with NO donors can often elicit the same responses induced by these hormonal or environmental stimuli. It is thus important to elucidate the processes that control NO synthesis and accumulation; however, in most cases, these processes are poorly understood (Meyer et al., 2005; Tischner et al., 2007; Neill et al., 2008a; Wilson et al., 2008; Gas et al., 2009; Leitner et al., 2009). In the case of hypoxia or anoxia, it is clear that nitrite serves as a substrate and is reduced to NO by nitrate reductase, mitochondria, or acid-catalyzed reactions (Yamasaki et al., 1999; Desikan et al., 2002; Rockel et al., 2002; Bethke et al., 2004; Tischner et al., 2004; Gupta et al., 2005; Planchet et al., 2005). Under normoxic conditions, however, the mechanisms and genes have not been resolved, but chloroplasts (Gas et al., 2009) and peroxisomes (Corpas et al., 2009) play a role.

NO has a strong relationship with another reactive species: hydrogen peroxide. Hydrogen peroxide works synergistically with NO to stimulate or delay programmed cell death and assist in defense responses to pathogens (Beligni et al., 2002; Wendehenne et al., 2004; de Pinto et al., 2006; Zaninotto et al., 2006; Asai et al., 2008; Besson-Bard et al., 2008; Asai and Yoshioka, 2009; Zhang et al., 2009). Hydrogen peroxide is also a signal for stomatal closure; ABA induces H_2O_2 synthesis, which, in turn, induces NO accumulation (Garcia-Mata and Lamattina, 2001, 2002; Desikan et al., 2004; Bright et al., 2006; Neill et al., 2008b). H_2O_2 plays a role in many other processes as well (Apel and Hirt, 2004; Mittler et al., 2004; Foyer and Noctor, 2005; Gapper and Dolan, 2006; Gechev et al., 2006; Pitzschke et al., 2006). The H_2O_2-responsive transcriptome has been determined (Desikan et al., 2001; Vanderauwera et al., 2005; Gadjev et al., 2006) and can be distinguished from the responses to other reactive oxygen species, such as singlet oxygen (Gadjev et al., 2006; Laloi et al., 2007).

To date, several genes that affect NO accumulation have been identified through genetic analysis. The *Arabidopsis thaliana NOA1* gene, which encodes a cGTPase (Moreau et al., 2008) and is needed for NO accumulation during abiotic and biotic

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responses (reviewed in Gas et al., 2009), was identified through reverse genetics (Guo et al., 2003). The *Arabidopsis nox1* mutant was identified in a screen for NO hypersensitive mutants and has a defective *CUE1* gene (encodes a chloroplast phosphoenolpyruvate/phosphate translocator), resulting in higher levels of NO and in delayed flowering (He et al., 2004). Arginase-negative mutants have increased NO accumulation and enhanced lateral root formation (Flores et al., 2008). Nitrate reductase mutants are defective in some NO-mediated processes, such as ABA-induced stomatal closure but are normal in others (reviewed in Gas et al., 2009). Several kinases in the mitogen-activated protein kinase cascade regulate NO bursts during defense responses in *Nicotiana benthamiana* (Asai et al., 2008; Asai and Yoshioka, 2009), and GPA1, a subunit of heterotrimeric G proteins, is necessary for *NOA1*-dependent NO accumulation that is stimulated by external calmodulin in stomates (Li et al., 2009). To identify additional genes that mediate and regulate NO synthesis and accumulation, we developed a genetic screen for NO-deficient mutants using the fluorescein dye 4-amino-5-methylamino-2′,7′-difluorescein (DAF-FM), which reacts with NO to produce a fluorescent compound. NO levels were measured after treatment with the physiological inducer H_2O_2. The isolation of a mutant defective in H_2O_2-induced NO accumulation is described below along with identification of the mutated gene and characterization of the NO-related phenotypes in the mutant.

RESULTS

Identification of a Mutant Defective in H_2O_2-Induced NO Accumulation

To develop a screen for mutants defective in NO accumulation, H_2O_2 was used as an inducer because it had been shown to elicit strong NO accumulation in plant leaves, stomates, and cell culture (reviewed in Neill et al., 2008b). To monitor NO accumulation, the NO-reactive dye DAF-FM diacetate (DAF-FM DA) was used. *Arabidopsis* roots were selected as the target organ due to the ease of H_2O_2 treatments and the advantage of measuring DAF fluorescence unimpeded by leaf or chloroplast fluorescence. We first verified that NO accumulation responds to H_2O_2 in *Arabidopsis* roots as described for other systems. Roots of 7-d-old seedlings treated with 100 μM H_2O_2 for 30 min showed an eightfold increase of DAF fluorescence (Figure 1A). Staining with the related fluorescein dye 4-AF DA, which does not react with NO, showed almost no increase in fluorescence in response to 100 μM H_2O_2 compared with DAF-FM DA (Figure 1B). Catalase, which degrades H_2O_2, or the NO scavenger cPTIO [2-(4-carboxyphenyl)-4,4,5,5 tetramethylimidazoline-1-oxyl-3-oxide] strongly reduced the H_2O_2 response (Figures 1C and 1D). The H_2O_2 generating system glucose/glucose oxidase also stimulated NO accumulation (Figure 1E), which was not observed with 4-AF DA or if catalase or cPTIO was included in the treatment (Figures 1F to 1H). These results indicate that H_2O_2 induces strong NO accumulation in *Arabidopsis* roots.

To screen for mutants defective in NO accumulation, ethyl methanesulfonate–mutagenized M2 seedlings were treated with DAF-FM DA and 100 μM H_2O_2. Approximately 40,000 M2 seedlings were screened. A seedling showing weak fluorescence (26-2) was identified then backcrossed twice with wild-type plants. Backcrossed progeny from 26-2 showed the same weak fluorescence as the parent (~40% the wild-type level; Figure 2) after 100 μM H_2O_2 treatment. Increasing the level of H_2O_2 resulted in recovery of DAF fluorescence to wild-type levels, reaching a level of 96% of wild-type at 1 mM H_2O_2 (Figure 2). To verify that the differences in DAF-FM fluorescence was due to changes in NO levels and not to differences in DAF-FM uptake or stability, wild-type and mutant seedlings

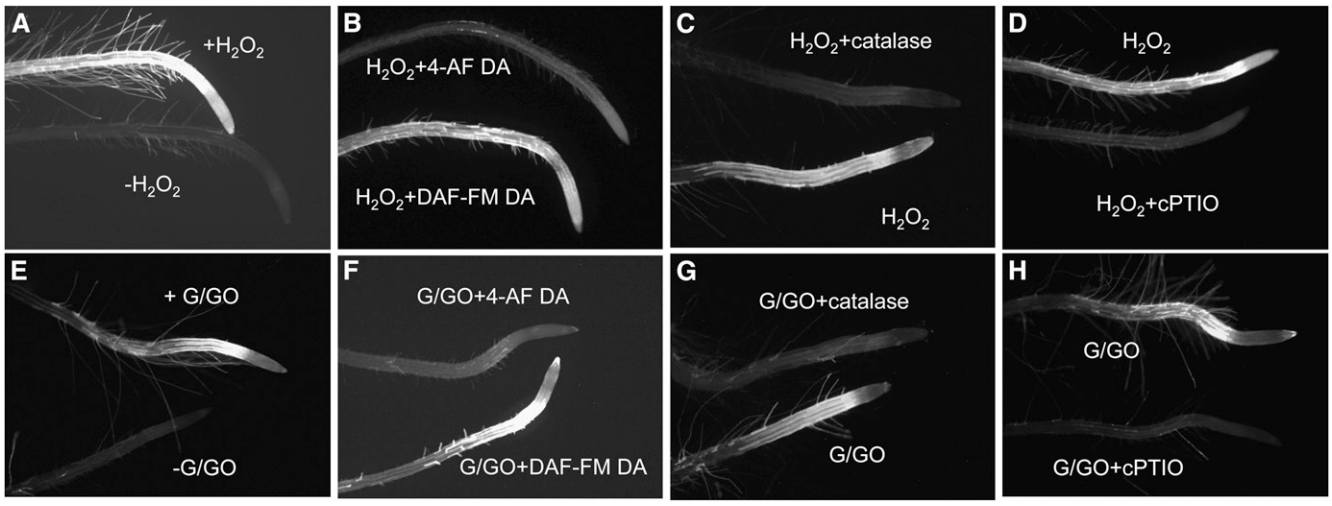

Figure 1. H_2O_2-Induced NO Accumulation in Wild-Type Roots.

The roots of 7-d-old seedlings grown on agarose half-strength Murashige and Skoog (MS) plates were treated for 30 min with 100 μM H_2O_2 (**[A]** to **[D]**) or with the H_2O_2-generating system 0.5 mM glucose 0.5 units/mL glucose oxidase (**[E]** to **[H]**). Catalase (100 units/mL; an H_2O_2 scavenger; **[C]** and **[G]**) or 500 μM cPTIO (an NO scavenger; **[D]** and **[H]**) was included as shown. NO production was detected using 10 μM DAF-FM DA, a fluorescent probe for NO.

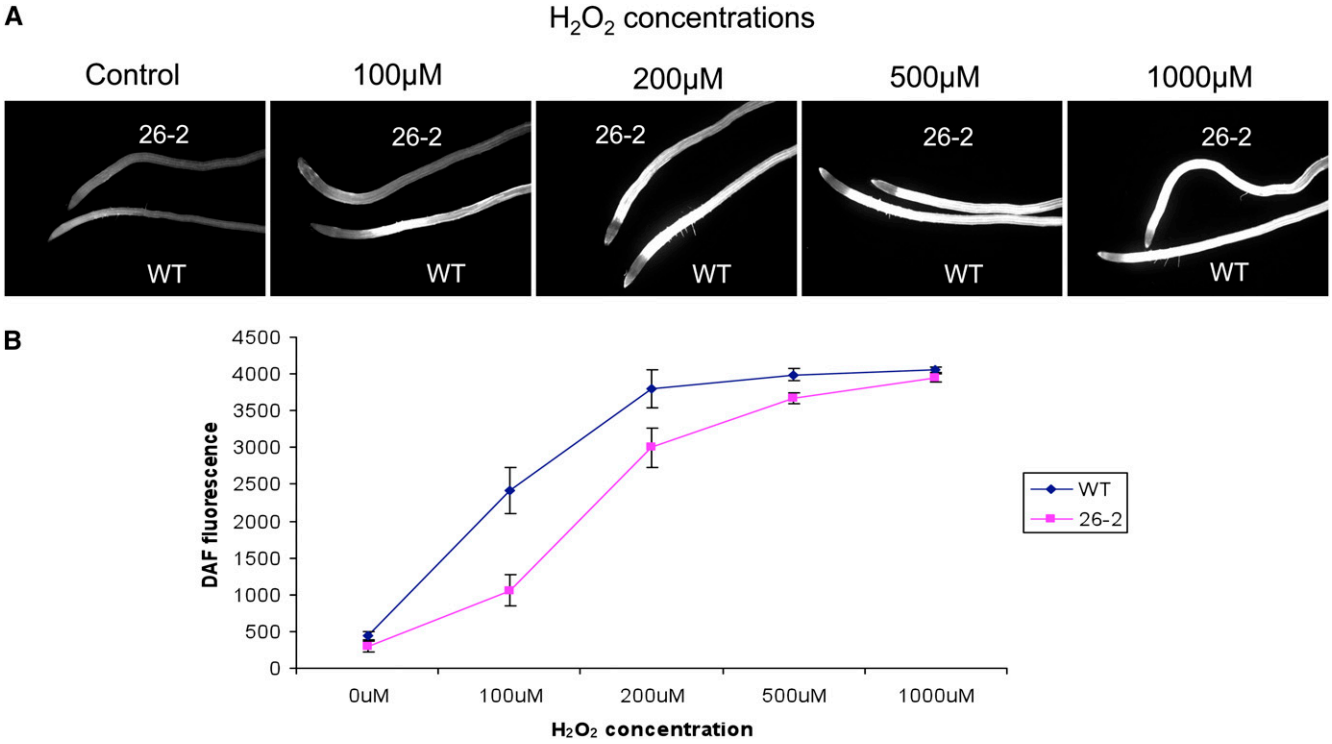

Figure 2. NO Levels in Wild-Type and 26-2 Mutant Seedling Roots after H_2O_2 Treatment.

(A) NO levels in the roots of 3-d-old wild-type and 5-d-old 26-2 seedlings (age where size is comparable) after 15 min of H_2O_2 treatment were detected using 10 μM DAF-FM DA.

(B) DAF-FM fluorescence signals from roots corresponding to treatments in **(A)** (n = 10) were quantified using the ImageJ program. Error bars represent SD (n = 10).

[See online article for color version of this figure.]

were stained with DAF-FM DA and then treated with the NO donor NOC9. Similar levels of fluorescence were observed in wild-type and mutant roots and cotyledons after this treatment (see Supplemental Figure 1 online), indicating that DAF-FM is functioning at equivalent levels in both lines. This mutant line was examined further to determine the identity of the gene that was mutated and uncover any additional NO-related phenotypes.

Mapping and Cloning of the Mutated Gene

During seedling growth, 26-2 shows significant growth retardation. At 12 d, wild-type plants have four to five true leaves, but 26-2 plants have no visible true leaves (see Supplemental Figure 2 online). This growth phenotype was used to map the 26-2 mutation using F2 progeny from a Landsberg *erecta* × 26-2 (Columbia) cross. The mutation mapped to the chromosome 5 in a region encompassed by BAC clones MNF13 and MHK7 (Figure 3). Genomic DNA of this region was cloned and sequenced from 26-2. A mutation within the gene At5g40770 was found that resulted in the conversion of Gly-37 to an Asp (Figure 3). Sequencing cDNA clones for At5g40770 from the mutant confirmed the presence of this mutation.

The gene At5g40770 encodes the *Arabidopsis* prohibitin protein PHB3 (Van Aken et al., 2007). Two other alleles of *PHB3* have been reported: *eer3-1*, which has an Asn substitution for Asp-165 (Christians and Larsen, 2007), and the loss-of-function allele *phb3ko* (SALK_020707) (Christians and Larsen, 2007; Van Aken et al., 2007), which contains a T-DNA insertion at the end of the first exon (Figure 3). RT-PCR using gene-specific primers revealed that the *PHB3* transcript was not detectable in the *phb3ko* mutant (see Supplemental Figure 3 online).

To verify that the mutation in *PHB3* was responsible for the reduced NO phenotype in 26-2, NO accumulation in *eer3-1* and *phb3ko* mutants was examined. Both mutants showed reduced fluorescence after treatment with 100 μM H_2O_2 (Figure 4). The *eer3-1* mutant showed the highest level of fluorescence among the mutants, and the *phb3ko* response was similar to 26-2. If one compares seedling growth, *eer3-1* shows the fastest growth, while 26-2 and *phb3ko* have the most stunted growth (see Supplemental Figure 2 online). These results indicate that mutations in *PHB3* reduce NO accumulation in response to H_2O_2 and that *eer3-1* appears to be the weakest allele, while 26-2 is a strong allele equivalent to a T-DNA insertion mutation. Hereafter, we refer to 26-2 as *phb3-3*.

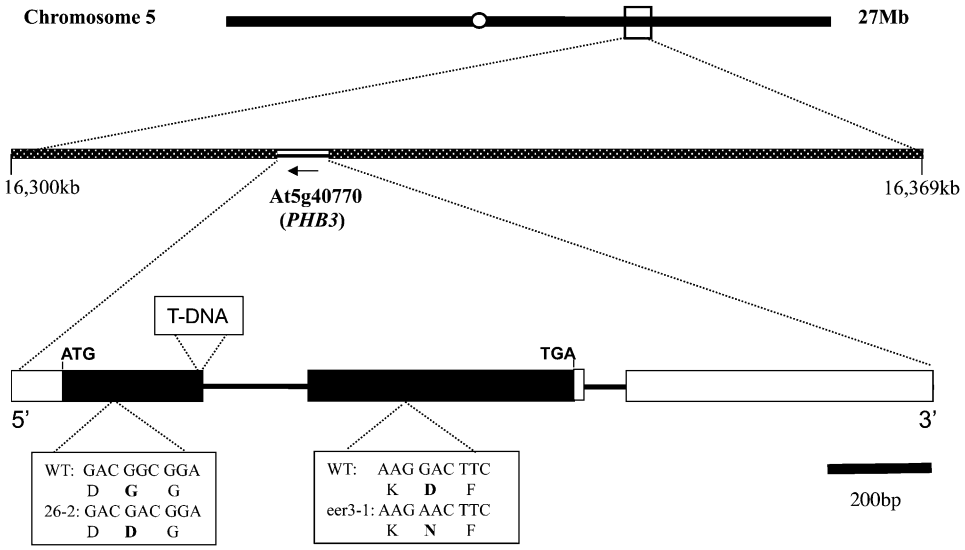

Figure 3. Mapping of Mutation 26-2 to *PHB3*.

The structure of the *PHB3* is shown with exons indicated by black boxes, introns by black line, and untranslated regions by white box. For the *phb3-3* mutant, the mutation is at nucleotide 111 of cDNA sequence, changing Gly-37 to Asp. For the *eer3-1* mutant, the mutation is at nucleotide 493 of cDNA, changing Asp-165 to Asn. The T-DNA insertion is for *phb3ko* line (Salk 020707).

H_2O_2-Induced Gene Expression and Accumulation Are Unaffected in the *phb3-3* Mutant

A possible explanation for the reduced accumulation of NO in response to H_2O_2 in the *phb3-3* mutant is that H_2O_2 signaling or accumulation is impaired so that the mutant has lower levels of or cannot respond to H_2O_2. To answer this question, the inductions of several H_2O_2-responsive genes were examined, including the WRKY transcription factor gene *WRKY6*, the heat stress transcription factor gene *HSFA4*, the touch gene *TCH3*, and the glutathione *S*-transferase gene *GSTU12* (Vanderauwera et al., 2005; Gadjev et al., 2006). Roots of 7-d-old seedlings grown hydroponically were treated with 0.5 mM H_2O_2 for 0.5 h, and the mRNA levels in roots for these genes were determined by quantitative RT-PCR. The results showed that all four genes were induced by H_2O_2, and the induction levels for each gene in *phb3-3* roots were similar to those in the wild type (Table 1). Next, H_2O_2 levels were examined using the fluorescent probe CM-H_2DCFDA [5-(and-6)-chloromethyl-2'7'-dichlorodihydrofluorescein diacetate acetyl ester]. Fluorescence was measured in both untreated roots and ABA-treated stomates. No difference in fluorescence between wild-type and mutant roots (see Supplemental Figure 4 online) or stomates was observed. From these data we find no evidence that *phb3-3* is defective in H_2O_2 sensing or accumulation and thus conclude that its phenotype is due to defects in NO accumulation.

ABA-Induced NO Production and Stomatal Closure Are Abolished in *phb3-3*

One of the best characterized, NO-mediated responses is ABA-induced stomatal closure. ABA enhances H_2O_2 production (Pei et al., 2000; Kwak et al., 2003), which stimulates guard cell NO accumulation and stomatal closure (Garcia-Mata and Lamattina, 2001, 2002; Desikan et al., 2004; Bright et al., 2006; Neill et al., 2008b). Because *phb3* mutants reduce H_2O_2-stimulated NO accumulation in roots, NO accumulation in guard cells and closure of stomates were examined in the mutant. Epidermal peels were prepared from wild-type and *phb3-3* plants and treated with ABA, and then NO levels and stomatal apertures were determined.

NO levels in wild-type stomates increased 2.7-fold when treated with 10 μM ABA (Figures 5A and 5B). No increase was observed for *phb3-3* mutant stomates after similar ABA treatment (Figures 5A and 5B). These data show that ABA-induced NO accumulation was severely inhibited in *phb3-3* guard cells. As described above, we found no evidence of reduced H_2O_2 accumulation in response to ABA using the fluorescent indicator dye CM-H_2DCFDA.

Stomatal closure was induced by ABA in wild-type stomates, which showed statistically significant reductions in aperture of 33 and 39% using 1 and 10 μM ABA, respectively, compared with control stomates (open bars, Figure 5C; $P < 0.001$). By contrast, ABA-induced stomatal closure was strongly inhibited in the *phb3-3* mutant. Mutant apertures were reduced by only 6 and 9% with 1 and 10 μM ABA treatments, respectively, which was not significant compared with untreated stomates (black bars, Figure 5C; $P > 0.05$). Measurements of aperture ratios confirmed these results. ABA treatments significantly decreased the ratios ($P < 0.001$) by 29 and 42% of control for 1 and 10 μM ABA treatments, respectively (Figure 5D); whereas, there were no significant differences in the ratios between ABA-treated and control stomates in *phb3-3* ($P > 0.05$; Figure 5D). These experiments demonstrate that *PHB3* is required for ABA-induced NO accumulation and stomatal closure.

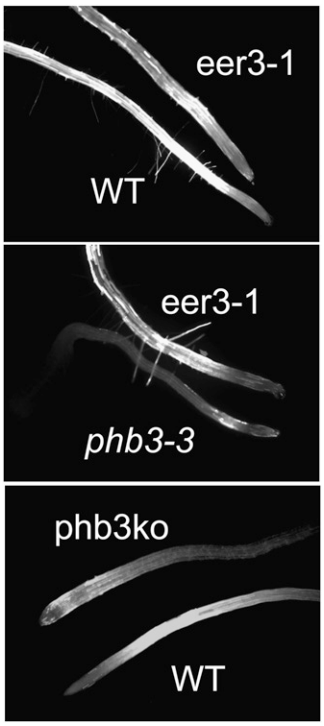

Figure 4. NO Levels in Wild-Type, *eer3-1*, and *phb3ko* Roots.

Seedlings were grown in liquid medium for 3 to 4 d and then treated with 100 μM H_2O_2 for 15 min. NO production in roots was detected with 10 μM DAF-FM DA.

Indole-3-Acetic Acid–Induced Lateral Root Formation Is Inhibited in the *phb3-3* Mutant

Auxin-induced lateral root formation is another well-studied, NO-mediated process (Pagnussat et al., 2002; Correa-Aragunde et al., 2004, 2006). To test *phb3-3* for defects in this auxin response, mutant and wild-type seedlings were treated with different concentrations of indole-3-acetic acid (IAA) for 3 d, and then the number of emerged lateral roots was determined. The experiment showed that lateral root number increased in a dose-dependent manner in wild-type roots (Figure 6). By contrast, lateral root number was unchanged in *phb3-3* roots for all IAA treatments tested, indicating IAA-induced lateral root formation is inhibited in *phb3-3*. Interestingly, without IAA treatment, *phb3-3* had a similar number of lateral roots as wild-type seedlings, showing that *phb3-3* was fully capable of producing lateral roots. To test if IAA sensing was impaired in the mutant, the induction of several IAA-responsive genes, including *IAA1*, *IAA5*, and *IAA19*, was examined. All three genes were induced to the same extent in both wild-type and *phb3-3* mutant roots after 0.5 and 1 h of 1 μM IAA treatment (Table 2), indicating that the mutant was still able to sense IAA and support rapid induction of gene expression.

NaCl Effects on NO Production and Primary Root Growth Were Impaired in the *phb3-3* Mutant

It has been reported that NO is involved in salt resistance as excess salt increases NO levels and salt stress can be alleviated by application of NO donors or be aggravated by lowering NO accumulation (Zhao et al., 2004, 2007; Corpas et al., 2009). Salt effects on the *phb3-3* mutant were tested by measuring NO accumulation and primary root lengths after treatment with NaCl. NO levels increased 2.7-fold in roots of wild-type seedlings after 0.5 h treatment with 200 mM NaCl (Figure 7A); by contrast, no change was observed in NaCl-treated *phb3-3* roots. When growth of primary roots was measured, mutant seedlings showed less inhibition from salt stress than wild-type seedlings (Figure 7B). After 2 d of 50 and 100 mM NaCl, the growth of wild-type roots was reduced by 45 and 69%, respectively, whereas root growth was reduced by only 33 and 46% in the mutant (Figure 7B).

The *phb3-3* Mutant Is Severely Defective in Green Light–Induced NO Accumulation in Cotyledons

Strong NO accumulation occurs when leaves are exposed to light (Gould et al., 2003). During our analysis of the *phb3-3* mutants, we noticed that the normal increase in DAF-FM fluorescence observed during green light exposure in cotyledons was drastically reduced in the *phb3-3* mutant compared with wild type (Figure 8). The cotyledons of 3-d-old wild-type and 5-d-old *phb3-3* mutant plants (ages that have similar sizes) were stained with DAF-FM DA and then exposed to green light from the fluorescent microscope. Fluorescence increased rapidly in wild-type cotyledons during the light exposure, saturating after 1 min. By contrast, no detectable increase in fluorescence was observed in mutant cotyledons except in stomates during light exposure (Figure 8). This effect was specific to cotyledons as true leaves show comparable increases in fluorescence in wild-type and mutant plants.

DISCUSSION

Our findings show a linkage between NO and prohibitin. Prohibitins are an extensively studied family of proteins that are highly conserved between animals and plants (Nadimpalli et al., 2000; Mishra et al., 2006). Prohibitins were first identified in a screen for regulators of human cell proliferation (McClung et al., 1989). Subsequently, prohibitins were shown to serve many functions in

Table 1. The Induction of H_2O_2-Responsive Genes in Wild-Type and *phb3-3* Roots after H_2O_2 Treatment

	WRKY6	HSFA4	TCH3	GSTU12
Wild type	7.1 ± 0.8	8.9 ± 0.7	27.6 ± 3.8	13.4 ± 2.3
phb3-3	7.9 ± 0.6	8.6 ± 0.8	25.0 ± 2.9	13.6 ± 1.7

The roots of 7-d-old seedlings grown hydroponically in half-strength MS were treated with 0.5 mM H_2O_2 for 0.5 h. RNA was extracted from the roots, and then mRNA levels were determined by quantitative RT-PCR. The gene UBA (ubiqitin-associated/TS-N domain-containing protein; At5g12120), which showed no response to H_2O_2 treatment, was used as an internal control. The four tested genes have the same mRNA levels in wild-type and *phb3-3* roots in the absence of H_2O_2. Fold induction values were determined as the ratio of treated versus control root mRNA levels with SD (n = 3).

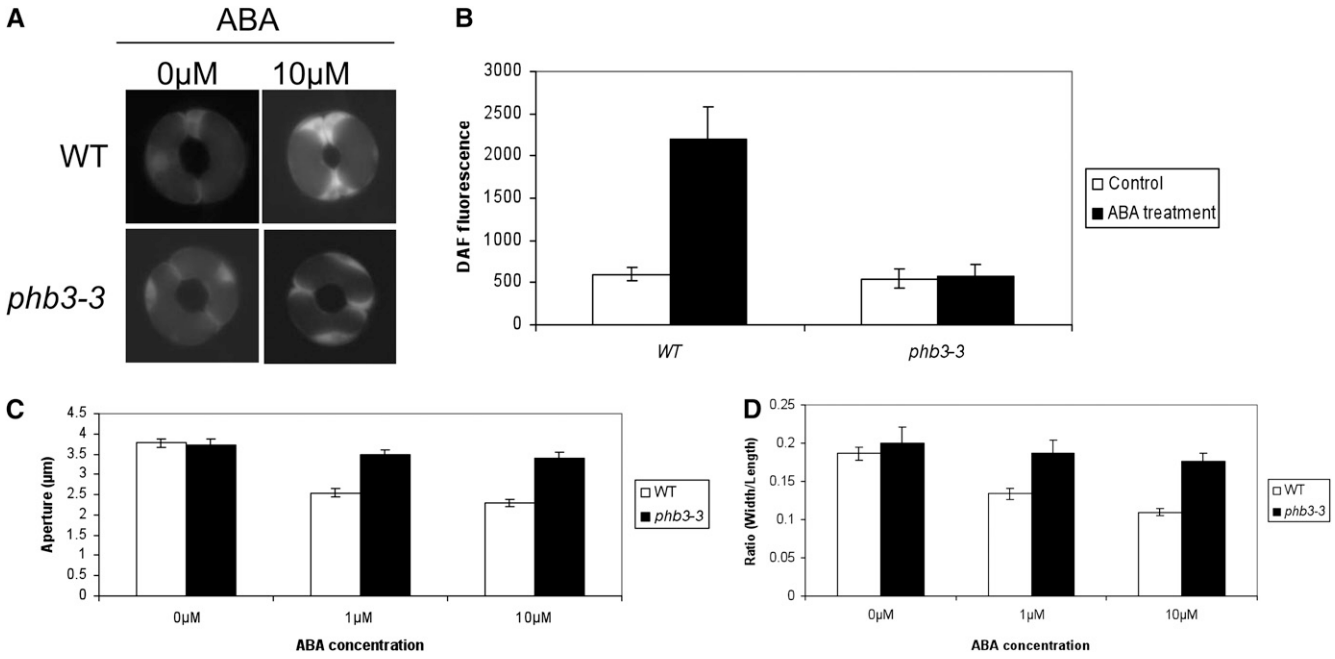

Figure 5. *PHB3* Functions in ABA-Induced NO Accumulation and Stomatal Closure.

Stomates prepared from leaves with similar sizes of 3-week old wild-type and *phb3-3* mutant plants were incubated for 2 h in stomate buffer and then treated with ABA.

(A) NO production in stomates treated with 10 μM ABA was detected by DAF-FM DA (5 μM).

(B) Relative fluorescence signals from guard cells corresponding to treatments in **(A)** were quantified using the ImageJ program.

(C) and **(D)** Apertures **(C)** and ratios of width/length **(D)** of stomates treated with 1 and 10 μM ABA are shown. Error bars represent SD (n = 40).

such diverse processes as apoptosis and aging, cell cycle progression, tumorigenesis, transcriptional regulation, signaling, oxidative damage, respiration, and mitochondrial biogenesis (reviewed in Mishra et al., 2006; Artal-Sanz and Tavernarakis, 2009). The biochemical function(s) of prohibitins is not clear, but they interact with receptors on the plasma membrane and with transcriptional regulatory components in the nucleus. In the mitochondria, PHB1 and PHB2 form a complex at the inner mitochondrial membrane and act as a chaperone that influences mitochondria ultrastructure, respiratory complexes, and cellular senescence (Artal-Sanz and Tavernarakis, 2009).

In plants, the initial identification and characterization of prohibitins showed a high degree of conservation with animal genes with >70% similarity at the amino acid level between plants and mammals and plants and yeast (Snedden and Fromm, 1997). Plant prohibitins play a role in plant defense (Nadimpalli et al., 2000), root hair elongation (Wen et al., 2005), cell division, development, and senescence (Chen et al., 2005; Ahn et al., 2006; Van Aken et al., 2007), oxidative stress (Ahn et al., 2006), and ethylene signaling (Christians and Larsen, 2007). Similar to animals, plant prohibitins are targeted to mitochondria, form multimeric complexes, and function in mitochondria biogenesis and function (Takahashi et al., 2003; Ahn et al., 2006; Van Aken et al., 2007).

Arabidopsis has seven members of the PHB family, which can be divided into two groups: the PHB1 group (PHB3, 4, and 5) and the PHB2 group (PHB1, 2, 6, and 7) (Ahn et al., 2006; Van Aken et al., 2007). The highest similarity among these proteins is between PHB3 and PHB4, which show 89% identity. Alignment of the *Arabidopsis* PHB proteins along with two *Saccharomyces cerevisiae* PHBs shows extensive similarity among these proteins (see Supplemental Figure 5 online), especially in the region containing the SPFH/band 7 domain, which is a common feature shared by a superfamily of proteins that form multimeric complexes and interact with membranes and the cytoskeleton (Browman et al., 2007; Langhorst et al., 2007; Hoegg et al., 2009). The mutation in *phb3-3* is at the beginning of the SPFH/band 7 domain of PHB3 (see Supplemental Figure 5 online).

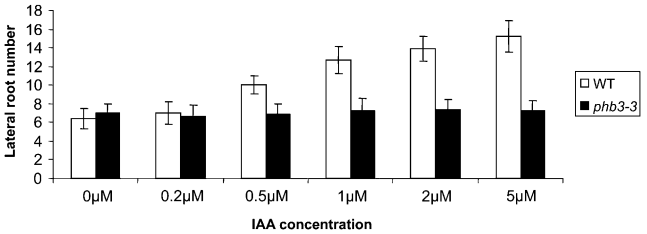

Figure 6. Auxin Effects on Lateral Root Formation in Wild-Type and Mutant Seedlings.

Three-day-old wild-type and 5-d-old *phb3-3* mutant seedlings (ages at which root length were equivalent) grown in liquid media were treated with different concentrations of IAA for 3 d. Number of visible lateral roots from each treatment is shown. Error bars indicate SD (n = 20).

Table 2. The Induction of IAA-Responsive Genes in Wild-Type and *phb3-3* Roots after IAA Treatment

IAA Treatment	IAA1	IAA5	IAA19
Wild type			
0.5 h	11.1 ± 2.8	18.2 ± 3.3	16.5 ± 3.7
1 h	16.8 ± 3.4	29.3 ± 3.5	38.9 ± 4.3
phb3-3			
0.5 h	9.6 ± 2.7	16.6 ± 2.6	19.4 ± 2.8
1 h	14.1 ± 2.7	26.6 ± 3.8	36.3 ± 2.9

The roots of 7-d-old seedlings grown hydroponically in half-strength MS were treated with 1 μM IAA for 0.5 and 1 h. RNA was extracted from the roots, and mRNA levels were determined by quantitative RT-PCR. The gene ACT2 (ACTIN2; At3g18780) showed stable expression when treated with IAA and was used as an internal control. The three IAA genes have the same mRNA levels in wild-type and *phb3-3* roots without IAA treatment. Fold induction values were determined as the ratio of treated versus control root mRNA levels with SD (n = 3).

Of the *Arabidopsis* prohibitin genes, *PHB3* is the most studied. It is expressed primarily in regions of active cell proliferation, including the root and shoot apices (Van Aken et al., 2007). It is induced by auxin and shows elevated expression in pericycle cells that give rise to lateral roots. *PHB3* knockout mutants show severe growth defects and have decreased cell division and expansion in the root apex. *phb3* mutants also have larger, rounder mitochondria. Interestingly, *phb4* single mutants display no phenotype (it is expressed at lower levels than *PHB3*), yet *phb3*/*phb4* double knockout mutants are not viable, suggesting that these two genes have compensating functions, with *PHB3* being the predominant gene. *PHB3* also functions in ethylene signaling (Christians and Larsen, 2007). It was identified in a screen for ethylene hypersensitive mutants and shows reduced hypocotyl length in the dark. The mutant, *eer3*, is an ethylene overproducer in the dark and has reduced induction of ethylene-responsive genes. The *eer3*/*phb3* mutation is epistatic to two ethylene insensitive mutations (*ein2* and *ein3*), indicating that *PHB3* functions downstream of these regulators (Christians and Larsen, 2007).

Our findings about *PHB3* provide a potential explanation for the diverse phenotypes reported for *phb3* mutants: *PHB3* regulates the level of NO accumulation and thus affects diverse processes involving NO signaling. For example, *PHB3* involvement in cell division and tissue proliferation (Van Aken et al., 2007) may be in part mediated via NO. For the case of auxin-induced lateral root formation, NO induces the cell cycle gene *CYCD3;1* and represses the inhibitory *KRP2* gene (Correa-Aragunde et al., 2006). This response could be reduced in *phb3* mutants due to lower levels of NO. Interestingly, it was recently reported that formation and elongation of adventitious roots in marigold *Targetes erecta* involves not only NO but also H_2O_2 (Liao et al., 2009), which supports the linkage between tissue proliferation and *PHB3*-mediated NO accumulation. There is also linkage between NO and ethylene. NO and ethylene levels are negatively correlated during fruit ripening (Leshem and Pinchasov, 2000), and NO has been shown to inhibit ethylene synthesis (Zhu and Zhou, 2007; Cheng et al., 2009). This linkage provides clues on how *phb3* mutations could produce the *eer3* phenotypes, which include higher ethylene production (Christians and Larsen, 2007). Results from a transcriptome study reported for *phb3* mutants are also consistent with our proposal. Many of the genes whose expression is altered by the *phb3* mutation are significantly upregulated by oxidative or salt stress as well as other abiotic stresses (Van Aken et al., 2007). Thus, some of the previously reported phenotypes resulting from *phb3* mutations can be explained, at least in part, by defects in NO accumulation.

Given what we know about prohibitins in general and *PHB3* in particular, we can speculate how *PHB3* affects NO accumulation and NO-mediated processes. The *phb3* mutations do not appear to affect H_2O_2 accumulation or signaling in our hands. Another article reported substantial increase in reactive oxygen species production and susceptibility in *Nicotiana* after suppressing *PHB* expression; however, this study used virus-induced gene silencing of *Nicotiana PHBs* (Ahn et al., 2006) so that it is difficult to compare with our findings. The result that *phb3* mutations reduce the level of NO at lower but not higher levels of H_2O_2 suggests that a simple loss of NO synthesis cannot explain the mutant phenotype. A more complex mechanism that affects rates of synthesis or degradation resulting in reduced NO accumulation in response to H_2O_2 is indicated. For example, disruption of mitochondrial function may result in decreased electron flux in the respiratory chain, resulting in less NO synthesis or more degradation. A model for this idea is the process of NO synthesis from nitrite via electron transfer through the respiratory

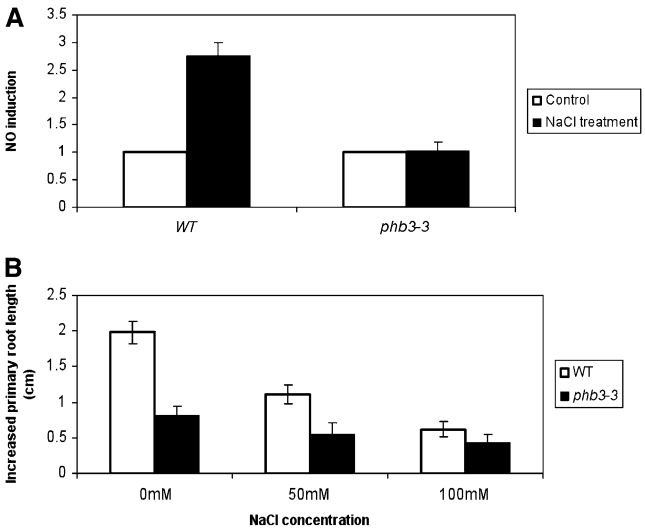

Figure 7. NaCl Effects on NO Accumulation in Roots and Primary Root Length of Wild-Type and Mutant Seedlings.

Three-day-old wild-type and 5-d-old *phb3-3* mutant seedlings grown in liquid media were treated with different concentrations of NaCl for 2 d.
(A) NO induction in wild-type and mutant roots was tested after 10 min treatment with 200 mM NaCl using DAF-FM DA. Induction ratios were determined as the ratio of treated versus control root levels.
(B) Increases in primary root length were calculated by subtracting primary root length at time zero from that measured after 2 d of the treatments. Error bars represent SD (**[A]**, n = 10; **[B]**, n = 20).

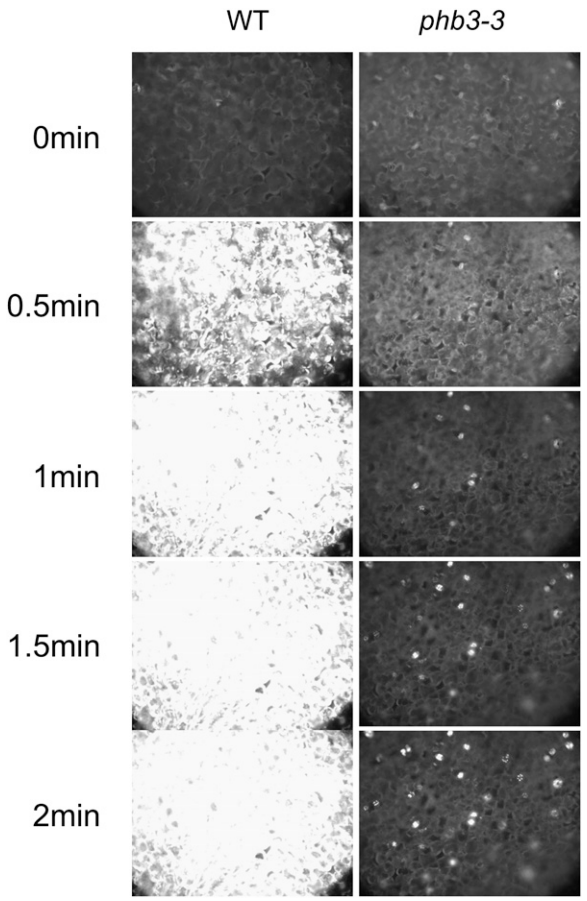

Figure 8. NO Accumulation in Wild-Type and Mutant Cotyledons under Green Light Treatment.

Wild-type and *phb3-3* mutant seedlings were grown in liquid media for 3 and 5 d, respectively. NO levels in cotyledons were detected using 10 μM DAF-FM DA, and photos were taken at the indicated time points after exposure to green light used for detecting DAF-FM fluorescence as described in Methods.

chain in root mitochondria during anoxia (Gupta et al., 2005; Benamar et al., 2008).

Lastly, our *PHB3* data provide further support for the involvement of NO signaling in developmental and abiotic stress responses in plants. NO accumulation in response to H_2O_2 is reduced in *phb3* mutants, and, at the same time, ABA-induced stomatal closure, auxin-induced lateral root formation, and salt-induced repression of primary root growth are also impaired. To elucidate this connection further, we will need to establish the mechanisms for NO synthesis in plants and the role prohibitins play in these mechanisms.

METHODS

Mutant Screen and Map-Based Cloning

Ethyl methanesulfonate–treated M2 *Arabidopsis thaliana* (Columbia) seeds (Lehle Seeds) were surface-sterilized and then placed in 6-well plates containing ~50 seeds and 1.5 mL half-strength MS medium (MS basal medium with vitamins [PhytoTechnology Laboratories], 0.5% [w/v] sucrose, and 0.5 g MES, pH 5.7) in each well. After 2 d of incubation at 4°C, seedlings were grown at 25°C under constant agitation (100 rpm) with continuous light. Three- to four-day-old seedlings were washed once with 2 mL half-strength MS medium per well and then treated with 1.5 mL half-strength MS medium and 10 μM DAF-FM DA (Molecular Probes) for 10 min. The seedlings were treated with 100 μM H_2O_2 for 10 min and then examined under a fluorescence microscope (Nikon Eclipse TE2000-U). Putative mutants were selfed and rescreened. Confirmed mutants were backcrossed to the Columbia wild type and made homozygous before analysis. Positional cloning of 26-2 (*phb3-3*) was performed on individual F2 recombinants using simple sequence length polymorphisms as described (Lukowitz et al., 2000). The *Arabidopsis phb3ko* line is a T-DNA insertion mutant (Alonso et al., 2003) obtained from Salk Institute Genomic Analysis Laboratory. The *eer3-1* mutant was a gift of Paul Larsen (University of California at Riverside).

NO Detection in Roots and Leaves

Seedlings grown in liquid half-strength MS medium for 3 to 5 d or on solid half-strength MS medium for 7 d were transferred to and incubated with 1.5 mL half-strength MS medium with 10 μM DAF-FM DA for 10 min before treating with H_2O_2, glucose/glucose oxidase, or NaCl. For cPTIO (Invitrogen) experiments, cPTIO was added to a concentration of 500 μM for 15 min before adding DAF-FM DA. Glucose/glucose oxide H_2O_2 generating system was set up using 0.5 mM glucose and 0.5 units of glucose oxidase, which produces a sustained level of 6 to 10 μM of H_2O_2 (Delledonne et al., 1998). For NO detection in leaves, wild-type and *phb3-3* seedlings were grown in liquid half-strength MS for 3 and 5 d, respectively. NO levels were measured in cotyledons using 10 μM DAF-FM DA and a green filter (B-2E/C FITC filter cube; Nikon).

H_2O_2 Detection in Roots

Wild-type and *phb3-3* seedlings were grown in liquid half-strength MS medium for 3 and 5 d, respectively, and then were transferred to 1.5 mL half-strength MS medium with 2 μM CM-H_2DCFDA (Invitrogen) for 10 min. For ascorbic acid treatment, ascorbic acid was added to a concentration of 2 mM for 20 min before adding CM-H_2DCFDA.

Gene Expression Analysis

For gene expression experiments, plants were grown under hydroponic conditions as previously described (Wang et al., 2003). Briefly, plants were grown in liquid half-strength MS medium at 25°C with continuous light. After 7 d, H_2O_2 was added to a final concentration of 0.5 mM and incubated for 0.5 h. For auxin treatments, 7-d-old seedlings were treated with 1 μM IAA for 0.5 and 1 h. Total RNA was extracted from roots using the RNeasy plant mini kit (Qiagen). Template cDNA samples were prepared as previously described (Wang et al., 2004). The cDNA synthesis reaction mixture was diluted 10-fold before being used for PCR. Real-time PCR was performed using LightCycler system from Roche Diagnostics.

Stomatal Experiments

Plants were grown on pots in soil at 25°C under 16 h light/8 h dark. Stomates were prepared from 3-week-old plant leaves, incubated for 2 h in stomatal opening buffer (5 mM MES, 10 mM KCl, and 50 μM $CaCl_2$, pH 6.15), and exposed to the indicated ABA concentrations as described (Vahisalu et al., 2008). After 2 h treatment, stomatal apertures were measured. For ABA-induced NO accumulation, 5 μM DAF-FM DA was used to detect NO levels in stomates after 0.5 h of ABA (10 μM) treatment.

For H_2O_2 measurements, 1 μM CM-H_2DCFDA was used after 0.5 h of ABA treatment. For all stomatal experiments, 40 stomates were tested per treatment, and three independent replicates were performed.

IAA and NaCl Effects on Root Growth

Three-day-old wild-type and 5-d-old *phb3-3* seedlings grown in liquid half-strength MS medium were treated with different concentrations of IAA and NaCl. Visible lateral roots were counted after 3 d of IAA treatment. Primary root length was measured at treatment start point and after 2 d of NaCl treatments, respectively, and increased primary root length was calculated by subtracting the first length from the second one. Twenty roots were measured for each treatment, and three replicates were performed.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative databases under the following accession numbers: At5g40770 (PHB3), At1g62300 (WRKY6), At4g18880 (HSFA4), At2g41100 (TCH3), At1g69920 (GSTU12), At5g12120 (UBA), At4g14560 (IAA1), At1g15580 (IAA5), At5g65670 (IAA9), and At3g18780 (ACT2).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. DAF-FM fluorescence in Wild-Type and Mutant Organs after Treating with a NO Donor.

Supplemental Figure 2. Growth Phenotype of 26-2 and Its Alleles.

Supplemental Figure 3. PCR Analysis of *PHB3* mRNA in *phb3ko* Lines.

Supplemental Figure 4. H_2O_2 Levels in Wild-Type and *phb3-3* Roots.

Supplemental Figure 5. Alignment of the *Arabidopsis* PHB Protein Family.

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