The Arabidopsis Mitochondria-Localized Pentatricopeptide Repeat Protein PGN Functions in Defense against Necrotrophic Fungi and Abiotic Stress Tolerance[1][C][W][OA]

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Pentatricopeptide repeat (PPR) proteins (PPRPs) are encoded by a large gene family in Arabidopsis (*Arabidopsis thaliana*), and their functions are largely unknown. The few studied PPRPs are implicated in different developmental processes through their function in RNA metabolism and posttranscriptional regulation in plant organelles. Here, we studied the functions of Arabidopsis *PENTATRICOPEPTIDE REPEAT PROTEIN FOR GERMINATION ON NaCl* (*PGN*) in plant defense and abiotic stress responses. Inactivation of *PGN* results in susceptibility to necrotrophic fungal pathogens as well as hypersensitivity to abscisic acid (ABA), glucose, and salinity. Interestingly, ectopic expression of *PGN* results in the same phenotypes as the *pgn* null allele, indicating that a tight regulation of the *PGN* transcript is required for normal function. Loss of *PGN* function dramatically enhanced reactive oxygen species accumulation in seedlings in response to salt stress. Inhibition of ABA synthesis and signaling partially alleviates the glucose sensitivity of *pgn*, suggesting that the mutant accumulates high endogenous ABA. Accordingly, induction of *NCED3*, encoding the rate-limiting enzyme in stress-induced ABA biosynthesis, is significantly higher in *pgn*, and the mutant has higher basal ABA levels, which may underlie its phenotypes. The *pgn* mutant has altered expression of other ABA-related genes as well as mitochondria-associated transcripts, most notably elevated levels of *ABI4* and *ALTERNATIVE OXIDASE1a*, which are known for their roles in retrograde signaling induced by changes in or inhibition of mitochondrial function. These data, coupled with its mitochondrial localization, suggest that PGN functions in regulation of reactive oxygen species homeostasis in mitochondria during abiotic and biotic stress responses, likely through involvement in retrograde signaling.

Plants display diverse survival mechanisms against microbial infection and other environmental stresses. While specialized host responses do occur, many components of the molecular events underlying plant responses to abiotic and biotic stresses are common. Passive defenses, such as the cuticle, aid in drought tolerance and protection from UV damage while also acting as a deterrent of herbivory and barrier against pathogen infection (Reina-Pinto and Yephremov, 2009). Similarly, the cellular and biochemical processes associated with active responses to different abiotic and biotic stimuli also share functional overlaps (Fujita et al., 2006). These induced responses are largely mediated by plant hormones and their interactions, which range from simple synergism or antagonism to intricate networks of cross-regulation (Grant and Jones, 2009). Responses to pathogen infection are modulated by salicylate (SA), jasmonate (JA), and ethylene (ET) with a growing role for abscisic acid (ABA), auxin, and GAs. ABA, a major regulator of environmental stress responses, is generally regarded as a negative regulator of plant defense, with exogenous application or increased endogenous levels typically correlating with plant susceptibility to pathogens (Mauch-Mani and Mauch, 2005; Fujita et al., 2006). However, there are instances of ABA positively contributing to disease resistance through modulation of callose deposition, stomatal closure, defense gene expression, and accumulation of reactive oxygen species (ROS; Mauch-Mani and Mauch, 2005).

Overall, plant responses to different stresses share significant overlap and points of convergence defined by regulatory factors that integrate signaling from various pathways (Fujita et al., 2006; Robert-Seilaniantz et al., 2010). Among these, the Arabidopsis (*Arabidopsis thaliana*) R2R3MYB transcription factor *BOS1* is a mediator of abiotic and biotic stress responses, its loss of function resulting in susceptibility to necrotrophic

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infection as well as hypersensitivity to salt, osmotic, and oxidative stress (Mengiste et al., 2003). Similarly, overexpression of the *ATAF1* transcription factor results in susceptibility to *Botrytis cinerea* and *Blumeria graminis* f. sp. *hordei* as well as decreased tolerance to ABA, salt, and oxidative stress (Jensen et al., 2007; Wu et al., 2009). *PHYTOCHROME AND FLOWERING TIME1* (*PFT1*) regulates plant resistance to *Alternaria brassicicola*, *B. cinerea*, and *Fusarium oxysporum* through its function in the biosynthesis of anthocyanin, a flavonoid linked to numerous abiotic and biotic stress responses (Kidd et al., 2009). *PFT1* encodes a subunit of the evolutionarily conserved Mediator complex, which was recently shown to promote transcription of *microRNA* (*miRNA*) genes by recruiting RNA polymerase II to their promoters (Kim et al., 2011). miRNAs and small interfering RNAs have emerged as important regulators of plant defense and stress tolerance known to affect gene expression, ROS accumulation, and plant cell death (Borsani et al., 2005; Katiyar-Agarwal et al., 2007; Sunkar et al., 2007; Xie and Qi, 2008). Natural cis-antisense small interfering RNAs have been associated with Arabidopsis salt tolerance as well as resistance to pathogens (Borsani et al., 2005; Katiyar-Agarwal et al., 2007; Xie and Qi, 2008). Natural small interfering RNA ATGB2 contributes to RPS2-mediated resistance to *Pseudomonas syringae* by repressing *PENTATRICOPEPTIDE REPEAT PROTEIN-LIKE* (*PPRL*) gene expression (Katiyar-Agarwal et al., 2007).

Members of the eukaryotic pentatricopeptide repeat (PPR) protein (PPRP) family contain tandem arrays of a degenerate 35-amino acid repeat and function in RNA or DNA modification through sequence-specific binding (Saha et al., 2007). As such, PPRPs have been associated with all stages of RNA processing, maturation, and translation (Saha et al., 2007; Schmitz-Linneweber and Small, 2008). PPRPs are classified into subgroups based on C terminus domains as well as the nature and order of their repeats (Small and Peeters, 2000; Lurin et al., 2004). Three conserved motifs, E-, E+, and DYW, in the C terminus define four subclasses of the PPRP family. These domains always require the one prior to be present in the protein; therefore, the subclasses consist of PPRPs with no C-terminal motifs, E-, E+ (preceded by E-), and DYW (preceded by E+ and E-; Lurin et al., 2004). The repeat motifs are defined as P, L, or S based on size and variability: P-type is the characteristic repeat defining the protein family, L-type is a long variant of the P repeat, and S-type is a short variant (Lurin et al., 2004). The Arabidopsis genome contains more than 450 PPRPs, yet surprisingly few have been studied, and their functions remain largely unknown (Schmitz-Linneweber and Small, 2008). The few studied PPRPs play diverse and crucial roles in plant growth and development, including embryogenesis, circadian rhythm, chloroplast development, and retrograde nuclear signaling (Lurin et al., 2004; Oguchi et al., 2004; Tzafrir et al., 2004; Cushing et al., 2005; Ding et al., 2006; Koussevitzky et al., 2007; Chi et al., 2008). GUN1, a DNA-binding chloroplast PPRP, is involved in retrograde signaling, regulation of *ABI4* expression, and photooxidative stress responses (Zhang et al., 2006; Koussevitzky et al., 2007). *ABI4* functions in light and sugar-induced stress responses as well as callose-mediated defense, ROS signaling, and resistance to fungal infection (Ton et al., 2009). LOVASTATIN INSENSITIVE1 (LOI1) is a PPRP that regulates biosynthesis of isoprenoids, metabolites known to affect defense gene expression in response to wounding and pathogen infection (Kishimoto et al., 2005; Kobayashi et al., 2007). The *loi1* mutant has decreased sensitivity to two inhibitors of isoprenoid synthesis, the fungal phytotoxin lovastatin and the herbicide clomazone, as evidenced by higher sterol and chlorophyll accumulation compared to that of treated wild-type plants (Kobayashi et al., 2007). PPR40 is a mitochondrial PPRP involved in oxidative respiration that also contributes to abiotic stress tolerance in Arabidopsis (Zsigmond et al., 2008). The *ppr40* mutant exhibits enhanced sensitivity to ABA and salinity that correlates with increased ROS accumulation and altered stress-responsive gene expression. Thus far, of the 450 predicted PPRPs, only GUN1, LOI1, PPRL, and PPR40 have been associated with Arabidopsis defense and/or stress tolerance (Katiyar-Agarwal et al., 2006; Kobayashi et al., 2007; Koussevitzky et al., 2007; Zsigmond et al., 2008). However, the functional link of many PPRPs in chloroplast and mitochondrial development and/or regulation suggests they may play a role in managing perturbations in cellular redox elicited by different types of stress (Lurin et al., 2004; Andres et al., 2007; Saha et al., 2007). Here, we describe the function of the Arabidopsis *PENTATRICOPEPTIDE REPEAT PROTEIN FOR GERMINATION ON NaCl* (*PGN*) in plant resistance to necrotrophic fungi and tolerance to abiotic stress.

RESULTS

Identification of the *PGN* Gene and Its Role in Resistance to Necrotrophic Pathogens

Previously, Arabidopsis *BOTRYTIS-INDUCED KINASE1* (*BIK1*) was found to play a contrasting role in plant defense, positively contributing to resistance against *B. cinerea* but functioning as a negative regulator of resistance against virulent *P. syringae* (Veronese et al., 2006). In an effort to further define the role of *BIK1* in defense and identify genes involved in resistance, we compared the genome-wide transcript profiles of wild-type and *bik1* plants prior to and following *B. cinerea* inoculation (Dhawan et al., 2009). From this, *AT1G56570* (designated *PGN*), encoding a PPRP, was identified as a potential BIK1 target due to its increased basal expression in the mutant and significant induction in wild-type plants following infection (Fig. 1A). Interestingly, Arabidopsis *nahG* lines have reduced *B. cinerea*-induced *PGN* expression (Fig.

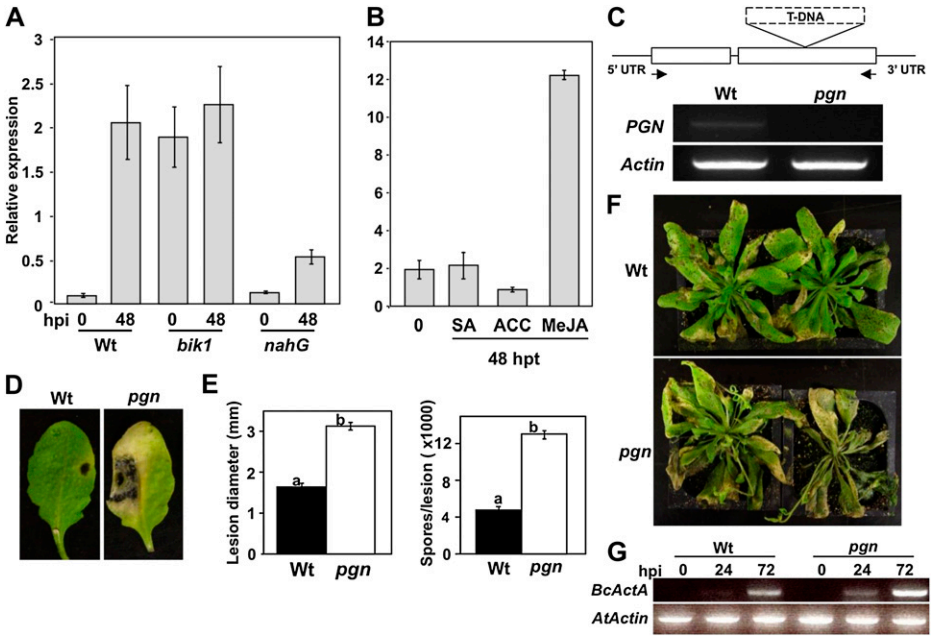

Figure 1. Expression of the *PGN* gene and characterization of the *pgn* mutant for disease resistance. A and B, Expression of *PGN* in response to *B. cinerea* (A) and exogenous application of plant hormones (B). C, Genomic organization of the *pgn* T-DNA insertion and loss of PGN expression (arrows indicate primer locations used to assay expression). D and E, Disease symptoms (D), lesion diameter (E), and number of spores per lesion 4 d after inoculation with *A. brassicicola*. F and G, Disease symptoms (F) and *B. cinerea ActinA* (G; *BcActA*) accumulation as a measure of fungal growth following spray inoculation with *B. cinerea*. Images were taken 5 (D) and 7 d after inoculation (F). Inoculation and quantification of disease symptoms/fungal growth were performed as described in "Materials and Methods." Data for lesion diameters and spores/lesion represent the mean ± SE from a minimum of 20 inoculated leaves. hpi, Hours postinoculation; hpt, hours posttreatment; UTR, untranslated region; Wt, wild type. Experiments were repeated at least three times with similar results.

1A). *nahG* plants are SA deficient, whereas the *bik1* mutation leads to high basal and induced SA accumulation (Delaney et al., 1994; Veronese et al., 2006), suggesting that *PGN* expression in response to *B. cinerea* is at least partially dependent on SA levels. Yet, exogenous application of SA does not affect *PGN* expression, whereas treatment with methyl-jasmonate (MeJA) leads to significant induction and the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC) causes minor suppression (Fig. 1B). Remarkably, *PGN* expression significantly correlates with the expression of 13 other *PPRP* genes as well as several genes associated with RNA synthesis or processing (Supplemental Fig. S1A; Obayashi et al., 2009). *PGN* is also highly expressed in dry and imbibed seed tissue as well as the shoot apex throughout development (Supplemental Fig. S1B).

To determine the biological relevance of its *B. cinerea*-induced gene expression, we characterized plants harboring a null T-DNA insertion allele of the *PGN* gene (*SALK_141937*; (Fig. 1C). The *pgn* mutation resulted in enhanced susceptibility to *A. brassicicola* as evidenced by increased chlorosis and necrosis at the site of inoculation (Fig. 1D). Compared to the wild type, *pgn* leaves develop significantly larger disease lesions and support increased fungal proliferation (Fig. 1E). The *pgn* mutant is also susceptible to *B. cinerea*. Following spray inoculation, *pgn* plants display increased chlorosis and tissue maceration 4 d after inoculation that progresses into abundant leaf decay around 7 d after inoculation (Fig. 1F). Despite relatively slow symptom development, fungal growth in the *pgn* mutant is higher than the wild type just 24 h after inoculation based on transcript accumulation of the constitutive *B. cinerea ActinA* gene (Fig. 1G).

Additionally, we assayed *pgn* plants for altered resistance to the bacterial pathogen *P. syringae* to further clarify *PGN* function in *BIK1*-regulated defense responses and determine if the altered susceptibility of the mutant to necrotrophic infection is a result of hormone-mediated defense antagonism. Plant immune responses to necrotrophic infection are regulated by JA/ET-mediated signaling events known to antagonize SA-dependent defenses associated with biotrophic resistance (Koornneef and Pieterse, 2008). No difference was observed in bacterial growth between wild-type plants and the *pgn* mutant inoculated with virulent (DC3000) or avirulent (DC3000AvrRpm1) strains of *P. syringae* (Supplemental Fig. S2). Interestingly, *B. cinerea*-induced expression of *PR-1* and *PDF1.2*, considered molecular markers of SA- and JA/ET-dependent defense responses, respectively, are not altered in the *pgn* mutant (Supplemental Fig. S3, A and B). Overall, these results suggest the function of *PGN* in

pathogen resistance is specific to necrotrophs and not associated with SA-, JA-, or ET-mediated defenses.

PGN Encodes a PPRP Localized to Mitochondria

PGN is predicted to encode a PPRP (Fig. 2A) with significant homology to proteins from different plant species and Arabidopsis spanning full-length amino acid sequences (Fig. 2B; Supplemental Fig. S4). The PGN protein shows the characteristics of the E+ PPRP subclass with a 43-S-P-L-S-P-L-S-34-L-S-1-P-L2-S-4-E-E+-19 repeat and motif arrangement (numbers indicate the amount of residues in between motifs; Fig. 2A). The repeats are predicted to form a superhelix compromised of two antiparallel α-helices able to bind DNA or RNA with substrate specificity dependent on the residue properties within the groove (Delannoy et al., 2007). In Arabidopsis, there are more than 450 predicted PPRPs, with all described proteins localized to mitochondria, chloroplast, or nucleus (Small and Peeters, 2000; Lurin et al., 2004; Schmitz-Linneweber and Small, 2008). Of the E+ PPRP subfamily, more than 40% are predicted to localize to mitochondria (Lurin et al., 2004). Prediction programs indicate PGN has a cleavable targeting sequence (MSITKLARSNA-FKPIPNFVRSSLRN) and a 96% likelihood of subcel-

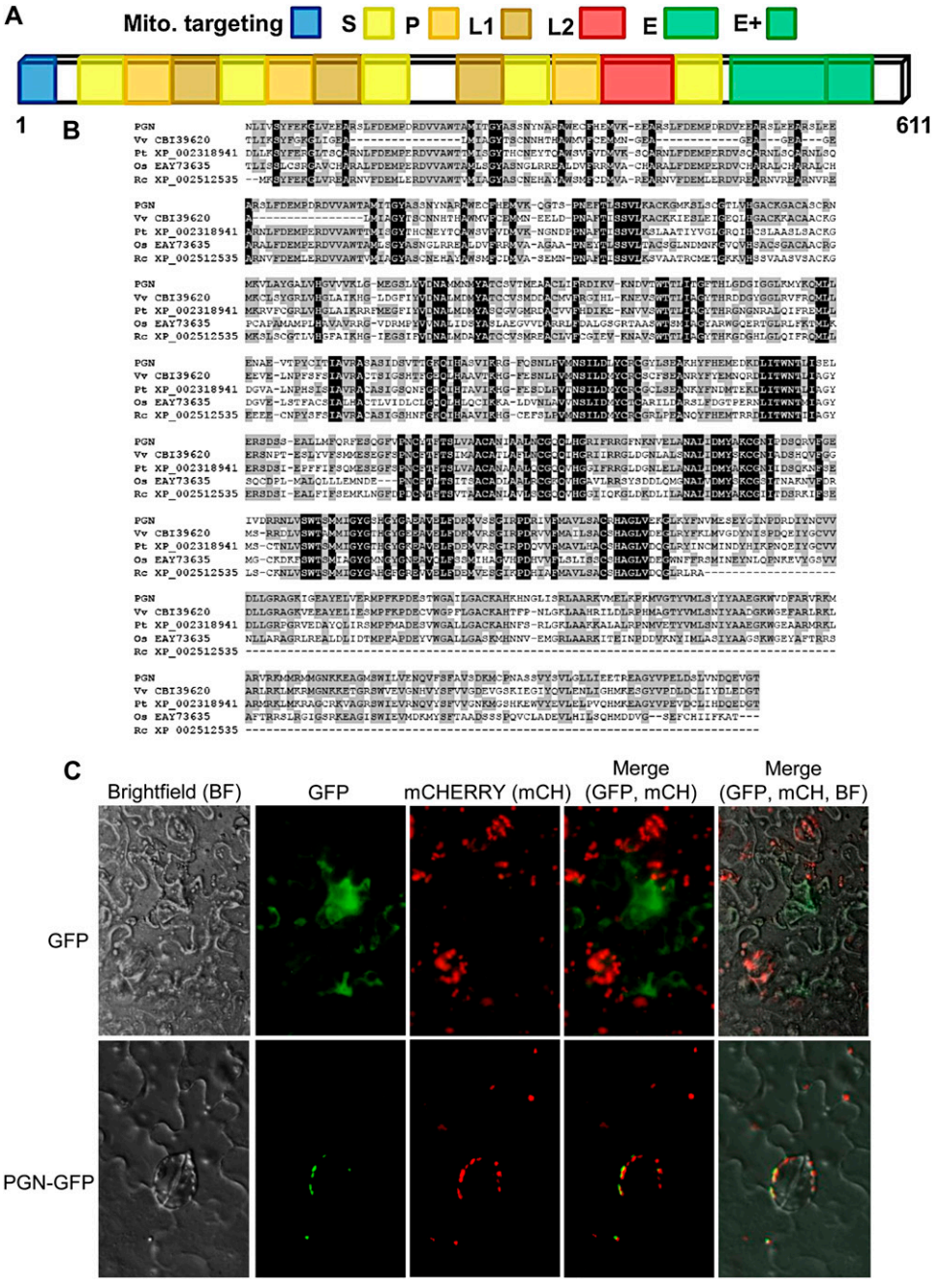

Figure 2. Main features of the PGN protein and its subcellular localization. A, Conserved domains of the PGN protein as defined by Lurin et al. (2004). B, Multiple sequence alignment of PGN and closely related proteins in other species. Black shading indicates conserved residues, and gray indicates residues identical to PGN. Proteins were aligned using ClustalW with default gap penalties (Thompson et al., 1994). C, PGN is localized to mitochondria. GFP, PGN-GFP, and the mitochondrial marker mCHERRY were transiently expressed in *N. benthamiana* leaf cells. Localization of tagged proteins was examined using the appropriate fluorescence wavelengths with images merged to show colocalization. Localization experiments were repeated at least three times with similar results. Vv, *Vitis vinifera*; Pt, *Populus trichocarpa*; Os, *Oryza sativa*; Rc, *Ricinus communis*.

lular localization to mitochondria (Claros and Vincens, 1996). To experimentally determine the subcellular localization of PGN, 35S:*PGN-GFP* was transiently expressed in *Nicotiana benthamiana* leaves in conjunction with different cellular markers. PGN-GFP colocalized with a mitochondrial marker (mCHERRY) consistent with the in silico predictions (Fig. 2C).

Loss or Gain of *PGN* Function Causes Hypersensitivity to ABA, NaCl, and Glc

To further study the function of *PGN*, overexpression (35S:*PGN;pgn*) and complementation lines (*PGNpr*:*PGN;pgn*) were generated (Fig. 3A). The different *PGN* genotypes were assayed for altered responses to hormones and abiotic stress agents in an effort to clarify the mechanism of *PGN* function in Arabidopsis defense. The *pgn* mutant displays germination hypersensitivity to media supplemented with ABA and increased Glc (Fig. 3, B–E). Overexpression of *PGN* also results in hypersensitivity to ABA and Glc comparable to that of the mutant (Fig. 3, B–E). Similarly, the *pgn* mutant and overexpression lines have increased sensitivity to salt, displaying increased salt-induced necrosis and chlorosis as well as reduced germination and root growth compared to wild-type seedlings (Fig. 4). Alternatively, both *pgn* and 35S:*PGN;pgn* plants have enhanced growth on Murashige and Skoog (MS) media lacking Glc relative to the wild type (Fig. 3B). In general, transformation with genomic *PGN* driven by its native promoter (*PGNpr*:*PGN;pgn*) restores mutant sensitivity to wild-type levels (Figs. 3, B–E, and 4). However, at higher concentrations of ABA, salt, and Glc, *PGNpr*:*PGN;pgn* seedlings have significantly reduced leaf emergence, root growth, and smaller cotyledons, respectively, relative to the wild type (Figs. 3, C–E, and 4, A, C, and D). This disparity could be attributed to the slightly higher *PGN* expression of these lines (Fig. 3A) compared to the wild type and further supports the hypersensitivity exhibited by 35S:*PGN;pgn* plants. Overall, these data suggest that, in addition to defense against necrotrophic infection, *PGN* regulates plant responses to abiotic stress, and its expression level is an important determinant of function. No altered seedling growth was observed when pregerminated 5-d seedlings were transferred to media supplemented with Glc, ABA, or indole-3-acetic acid (Supplemental Fig. S5, A–C). Also, germination on media supplemented with ACC, MeJA, SA, GA, indole-3-acetic acid, or hydrogen peroxide (H_2O_2) is not affected by the *pgn* mutation, limiting the role of *PGN* to a specific subset of stress and hormone responses (Supplemental Fig. S5D).

The Glc Hypersensitivity of the *pgn* Mutant Is Partially Restored by ABA Antagonists

Increased concentrations of Glc are known to delay Arabidopsis germination, with ABA levels determining the severity of inhibition (Gazzarrini and McCourt, 2001; Dekkers et al., 2004). Many mutants exhibiting enhanced growth on media containing high exogenous sugars are also ABA insensitive, whereas even minimal ABA increases act additively to sugar-mediated seedling growth inhibition (Laby et al., 2000; Gazzarrini and McCourt, 2001; Gibson et al., 2001). To determine if *pgn* hypersensitivity to Glc and ABA is a result of increased endogenous ABA, we assayed growth responses to 6% Glc in the presence of two ABA inhibitors: the ET precursor ACC and norflurazon (NF). ET antagonizes ABA function in germination, likely promoting emergence through suppression of ABA signaling and synthesis initiated by Glc and ABA (Beaudoin et al., 2000; Ghassemian et al., 2000; León and Sheen, 2003; Matilla and Matilla-Vazquez, 2008). NF inhibits ABA accumulation through disruption of carotenoid biosynthesis upstream of ABA biosynthesis (Bartels and Watson, 1978; Zeevaart and Creelman, 1988). Addition of ACC or NF partially relieved *pgn* hypersensitivity to Glc relative to 6% Glc alone (Fig. 5A). Interestingly, whereas ACC restored *pgn* growth to a degree comparable to respective wild-type controls, the effect of NF resulted in seedlings nearly half the size of corresponding wild type. These data suggest that the increased Glc sensitivity exhibited by the *pgn* mutant is likely due to high levels of endogenous ABA. Subsequent analysis of total ABA content indicates that *pgn* seedlings do have significantly higher basal levels of ABA compared to the wild type (Fig. 5B). The growth variation between treatments may be explained by disparities in the mechanism by which they inhibit ABA responses. ACC suppresses ABA accumulation and signaling, whereas NF only disrupts ABA biosynthesis. Thus, based on its phenotypes, the *pgn* mutant may be altered in ABA levels as well as signaling. Alternatively, variation may be a result of secondary effects of treatment, such as NF inhibition of GA synthesis. However, based on the normal responses of the mutant to GA and other plant hormones (Supplemental Fig. S5D), we limited our conclusions to the effects of these chemicals on ABA responses for which *pgn* is clearly altered. The independent restoration of growth responses with either ACC or NF also serves to further strengthen an ABA-dependent mechanism. Interestingly, low levels of Glc also have an inhibitory effect on germination, yet the effect is lessened in ABA-sensitive and -insensitive mutants (Garciarrubio et al., 1997; Price et al., 2003). Thus, the enhanced growth of *pgn* on media lacking Glc lends further support to its increased sensitivity to ABA (Fig. 5A).

Altered *PGN* Expression Enhances the Triple Response

Generally, ABA and ET function antagonistically, with most ET-insensitive mutants showing sensitivity to ABA and vice versa (Gazzarrini and McCourt, 2001). Our data suggest that *pgn* mutation leads to disrupted ABA responses that can be partially restored through ET-mediated antagonism (Fig. 5). This

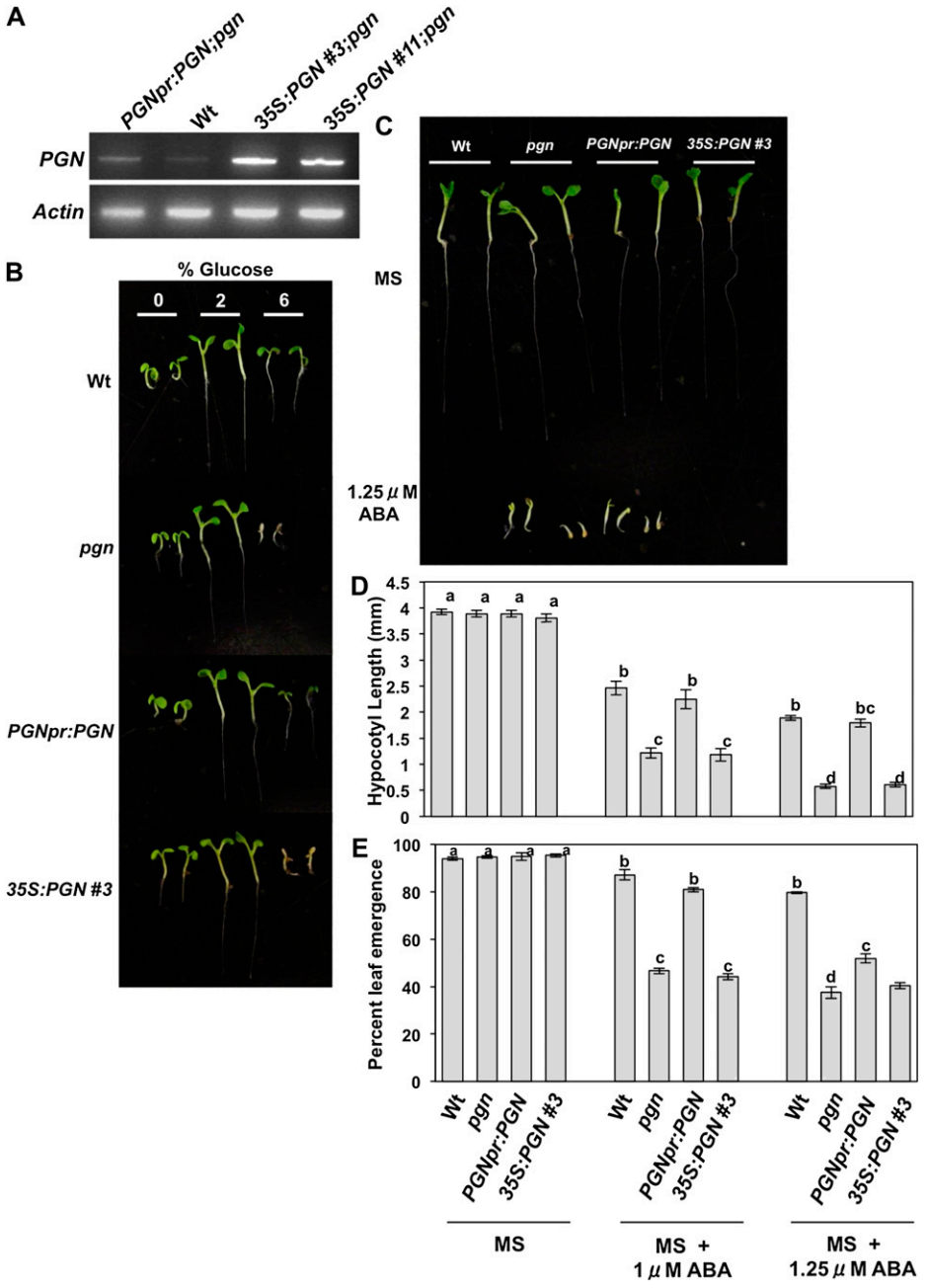

Figure 3. Loss or gain of *PGN* function causes hypersensitivity to Glc and ABA at germination. A, *PGN* expression in wild-type (Wt), complemented (*PGNpr:PGN;pgn*), and overexpression (35S:*PGN;pgn*) lines. B and C, Seedling growth of transgenic *PGN* lines on Glc (B) and ABA-supplemented media (C). D and E, Mean hypocotyl length (D) and percentage of leaf emergence (E) on ABA-supplemented media. Data represent the mean ± SE from a minimum of 40 seedlings. Experiments were repeated at least three times with similar results.

implies that loss of *PGN* function is likely to cause insensitivity to ET. However, instead of increased tolerance, the *pgn* mutant exhibits enhanced ET sensitivity as observed by an altered triple response (Fig. 6). The triple response is induced when plants are grown in the dark on media containing ET or its precursor ACC, producing seedlings with exaggerated apical hooks, swollen hypocotyls, and inhibited root/hypocotyl elongation (Bleecker et al., 1988). In response to ACC, *pgn* seedlings produce shorter hypocotyls with limited root growth compared to the wild type (Fig. 6). Ectopic *PGN* expression also results in an enhanced triple response comparable to that of the mutant, consistent with either loss or gain of *PGN* function resulting in the same phenotypes.

The *pgn* Mutant Has Altered ABA-Related Gene Expression

Loss of *PGN* function results in hypersensitivity to ABA as well as salt and Glc. To determine if this decreased tolerance is due to altered ABA signaling, we assayed the mutant for altered expression of ABA-responsive marker genes associated with activation of abiotic stress responses. Salt-induced expression of *RD29A*, *RAB18*, and *COR6.6* is not altered by the *pgn*

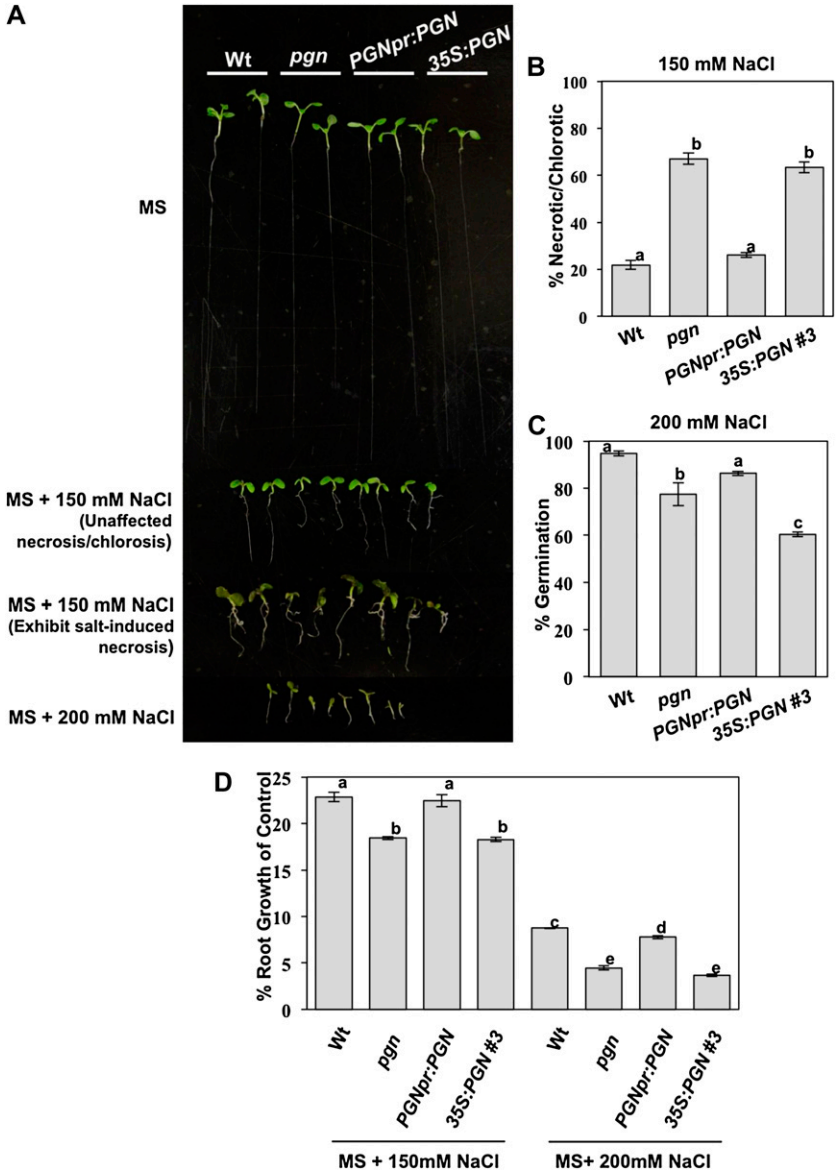

Figure 4. Altered *PGN* expression causes impaired germination and growth responses to increased salinity. A, Comparison of seedling growth on NaCl-supplemented media. The representative growth phenotypes of wild-type (Wt) and *pgn* seedlings on 200 mM NaCl (fourth row), exhibiting salt-induced necrosis and/or chlorosis on 150 mM NaCl (third row), and those that did not on 150 mM NaCl (second row) are presented. B, Percentage of seedlings exhibiting salt-induced chlorosis and/or necrosis on 150 mM NaCl. C, Percentage of germination on 200 mM NaCl. D, Percentage of reduction in root growth on NaCl relative to MS controls. Data represent the mean ± SE from a minimum of 40 seedlings. Experiments were repeated at least three times with similar results.

mutation (Supplemental Fig. S3C). *RD29A*, *RAB18*, and *COR6.6* are ABA-responsive genes with varying roles in salt, sugar, and osmotic stress tolerance (Mahajan and Tuteja, 2005; Chinnusamy et al., 2006). By contrast, *ABF3*, *DREB1B*, and *DREB2B* are all induced at lower levels in the *pgn* mutant in response to salt or ABA (Fig. 7, A and B). Salt-induce.d *ABI5* expression is also slightly reduced in *pgn*, but induction by ABA was unaffected by mutation (Fig. 7, C and D). Interestingly, *pgn* has higher *NCED3*, *EIN5*, and *ABI4* expression in response to both ABA and NaCl compared to the wild type (Fig. 7, C and D). *ABI4* is the most significantly affected by *pgn* mutation, showing nearly 5 times greater expression in the mutant compared to wild-type *ABI4* induction in response to ABA (Fig. 7C). *ABI4* encodes a transcription factor involved in ABA signaling and functions downstream of the PPRP GUN1 in photooxidative stress responses (Berrocal-Lobo et al., 2002; Koussevitzky et al., 2007).

B. cinerea-Induced *PGN* Expression Is Enhanced in the ET and ABA Response Mutants *etr1* and *abi5*

To further define *PGN* function in resistance to necrotrophic infection with respect to ABA and ET signaling, we examined its expression in mutants with altered ABA and ET responses. In response to *B. cinerea*, *PGN* expression was not altered in *etr1* or *abi1* (Fig. 8A). Mutation in the ET receptor *ETR1* results in significantly decreased *ABI1* expression, which is consistent with our results and suggests neither of these genes contribute to regulation of *PGN* during

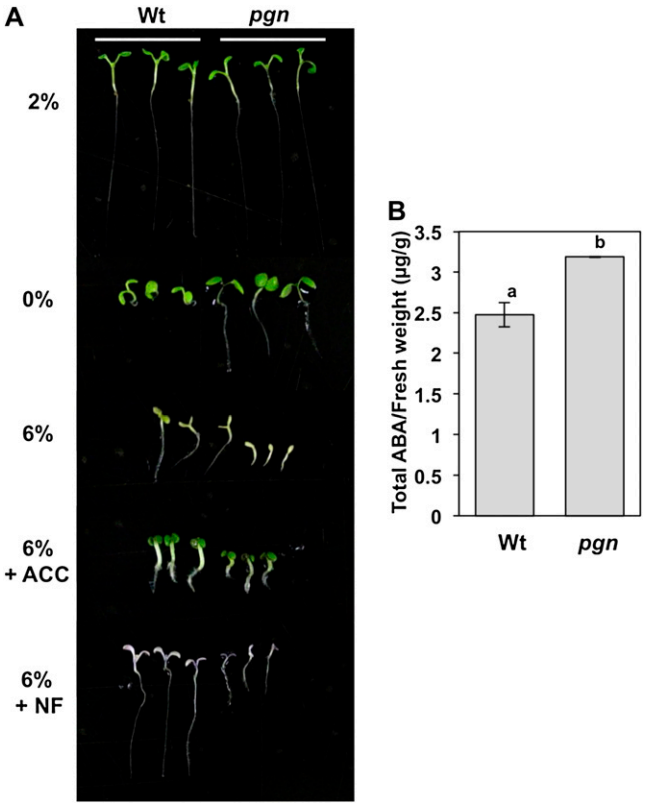

Figure 5. The Glc sensitivity of the *pgn* mutant is partially reversed by ACC and NF. A, Germination responses of wild-type (Wt) and *pgn* seedlings on varying concentrations of Glc and 6% Glc supplemented with ACC or NF. B, ABA content of 10-d-old seedlings determined by liquid chromatography-tandem mass spectrometry. Experiments were performed as stated in "Materials and Methods" and repeated at least two times with similar results.

infection. By contrast, inoculated *abi5* and *erf1* mutants exhibit markedly higher *PGN* induction. *ERF1* is a positive regulator of JA/ET-mediated defenses that functions partially through antagonism of ABA responses (Hildmann et al., 1992; Lorenzo et al., 2004). Thus, *PGN* expression may increase as ABA responses are relieved from *ERF1*-mediated repression. However, mutation in *ABI5*, a positive regulator of ABA signaling, also causes increased induction, suggesting that altered expression in these mutants may be linked to a secondary effect or that *PGN* regulation is not solely dependent on ABA responses (Berrocal-Lobo et al., 2002). In addition, basal expression of *PLEIOTROPIC REGULATORY LOCUS1* (*PRL1*) is significantly repressed in the *pgn* mutant (Fig. 8B). *PRL1* encodes a WD-40 protein that functions in global regulation of sugar, stress, and hormone responses as well as basal defense in Arabidopsis (Németh et al., 1998; Palma et al., 2007). Based on literature searches, *prl1* was the only mutant we could identify with increased sensitivity to Glc, ABA, and ET similar to that observed for *pgn*.

The *pgn* Mutation Decreases Chlorophyll Content in Seedlings But Does Not Impair Oxidative Stress Tolerance

Recently, the *PPR40* gene was isolated from a screen for ABA-sensitive Arabidopsis mutants (Zsigmond et al., 2008). In addition to ABA, the *ppr40* mutant is also sensitive to salt and oxidative stress. *PPR40* encodes a mitochondrial PPRP important for the function of Complex III in electron transport (Zsigmond et al., 2008). Due to the remarkable similarity of stress response phenotypes and shared subcellular localization of PGN and PPR40, we studied their functional relationship. The *ppr40* mutant has significantly higher basal *PGN* expression that becomes repressed after *B. cinerea* infection (Fig. 8C). This pattern of expression is in direct contrast to that in wild-type plants, suggesting that *PPR40* may function in the regulation of *PGN* transcription. Interestingly, *ppr40* mutation results in a loss of resistance to *B. cinerea* comparable to that resulting from loss of PGN function (Fig. 8D).

We also assayed the *pgn* mutant for tolerance to oxidative stresses. Detached leaf treatment with methyl viologen (MV), an herbicide that generates ROS, did not indicate any impaired oxidative stress tolerance in the *pgn* mutant (Supplemental Fig. S6A). Additionally, there was no significant difference in chlorophyll content between *pgn* and wild-type seedlings treated with H_2O_2 or MV as observed for the *ppr40* mutant (Zsigmond et al., 2008; Supplemental Fig. S6B). However, untreated *pgn* seedlings had significantly lower total chlorophyll content compared to the wild type. This difference was not observed in 4-week-old plants (Supplemental Fig. S6C). Thus, it appears that the *pgn* mutation does not affect oxidative stress tolerance in response to MV or H_2O_2, though it does lead to reduced chlorophyll levels in seedlings. Thus, PPR40 and PGN appear to have overlapping as well as distinct functions in Arabidopsis stress and defense responses.

The *pgn* Mutation Affects Mitochondrial Retrograde and Electron Transport Gene Expression

PGN's localization to mitochondria and putative function in RNA metabolism prompted us to assay expression of mitochondria-associated transcripts in the mutant. *pgn* has significantly higher salt-induced *ALTERNATIVE OXIDASE 1a* (*AOX1a*), *AOX1b*, *AOX1d*, *UCP*, *CCB45*, and *COX2* expression, with *AOX1d*, *UCP*, and *COX2* also showing increased basal expression (Fig. 9, A and D). The AOX proteins and UCP serve as nonphosphorylating bypasses of mitochondrial electron transport, whereas CCB45 and COX2 are associated with cytochrome *c* biogenesis and oxidation, respectively (van Dongen et al., 2011). *AOX* induction also serves as a marker of mitochondrial retrograde responses in Arabidopsis. No significant differences in expression were observed for the extensively edited mitochondrial *ORFX* transcript, *NAD2*, *NDB3*,

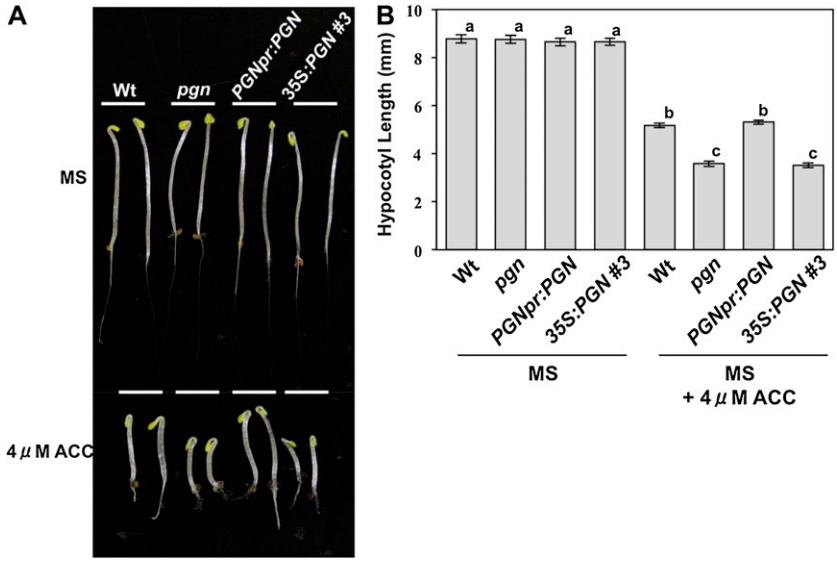

Figure 6. Loss or gain of *PGN* function enhances the triple response. Triple response phenotypes (A) and mean hypocotyl lengths (B) of the *PGN* genotypes following growth in the dark on ACC-supplemented media. Data represent the mean ± SE from a minimum of 40 seedlings. Experiments were repeated at least three times with similar results. Wt, Wild type. [See online article for color version of this figure.]

or *NDB4* encoding a protein of unknown function, an NAD(P)H dehydrogenase subunit, and NAD(P)H dehydrogenases B3 and B4, respectively (Fig. 9, B–E). Yet, the *pgn* mutant has significantly higher basal expression of the *NAD1* transcript, which is trans-spliced by the PPRP OTP43 and encodes an NAD(P)H dehydrogenase subunit associated with mitochondrial respiratory chain complex I (de Longevialle et al., 2007). *RPL2*, encoding a constituent of the large subunit of the mitochondrial ribosome, is also elevated in the mutant but gets repressed following salt treatment (Fig. 9B). *NAD9* and *MATR* are expressed at lower levels in *pgn* both before and after treatment, with the RNA maturase gene *MATR* showing the highest degree of suppression caused by *pgn* mutation (Fig. 9, B and E).

Altered ROS Metabolism in the *pgn* Mutant Contributes to Its Susceptibility to *B. cinerea* and Hypersensitivity to Salt

Consistent with germination assays in which both high and low *PGN* levels led to increased sensitivity to ABA, salt, and Glc, *PGN* overexpression resulted in enhanced susceptibility to *B. cinerea* similar to that observed for the mutant (Fig. 9F). Infected wild-type and complemented (*PGNpr*:*PGN*;*pgn*) plants shared similar levels of resistance without any observable differences in disease symptoms. Thus, the level of *PGN* expression appears to influence the extent of plant susceptibility to *B. cinerea*, consistent with results obtained for germination responses to abiotic stress agents. However, the threshold for transcript levels that alter responses appears to be lower for germination as the marginal increase in *PGNpr*:*PGN*;*pgn* plants results in weak sensitivity to abiotic stresses but has no effect on resistance to *B. cinerea*.

To determine if altered mitochondrial function in management of ROS is a contributing factor in *pgn* susceptibility, the *PGN* genotypes were inoculated with *B. cinerea* and incubated under constant light or dark. Under dark conditions, *pgn* and the overexpression line (35S:*PGN*;*pgn*) had significantly enhanced susceptibility relative to infected wild-type and complemented (*PGNpr*:*PGN*;*pgn*) plants as well as all plants incubated in light (Fig. 9F). Although light-grown plants maintained susceptibility, continuous darkness significantly enhanced disease symptoms. Thus, *pgn* susceptibility appears to be linked to impaired *B. cinerea*-induced ROS detoxification as, under dark conditions, this process becomes largely dependent on mitochondrial functions. The effect of *pgn* mutation on ROS metabolism was further confirmed by histochemical staining for the presence of H_2O_2 using 3,3′-diaminobenzidine (DAB) in salt-treated seedlings. Whereas minimal increases in H_2O_2 were observed in the leaf margins of wild-type seedlings, leaves of *pgn* seedlings incubated in 200 mM NaCl were intensely and wholly stained (Fig. 9G).

The *pgn* Mutation Does Not Affect Retrograde Signaling between the Chloroplast and Nucleus in Response to Photooxidative Stress

Recent studies have shown that GUN1 is a DNA-binding PPRP involved in retrograde signaling from the chloroplast (Koussevitzky et al., 2007). The *gun1* mutant exhibits higher *LIGHT HARVESTING CHLOROPHYLL BINDING1* (*LHCB1*) expression than the wild type after treatment with NF or lincomycin (Koussevitzky et al., 2007). Both chemicals disrupt chloroplast protein biosynthesis leading to Mg-ProtoIX accumulation (Koussevitzky et al., 2007). Mg-ProtoIX acts as a signal repressing nuclear expression of photosynthetic genes, specifically the *LHCB* family (Strand et al., 2003). Many of the genes affected by *gun1* mutation are involved in ABA signaling, with *ABI4* acting

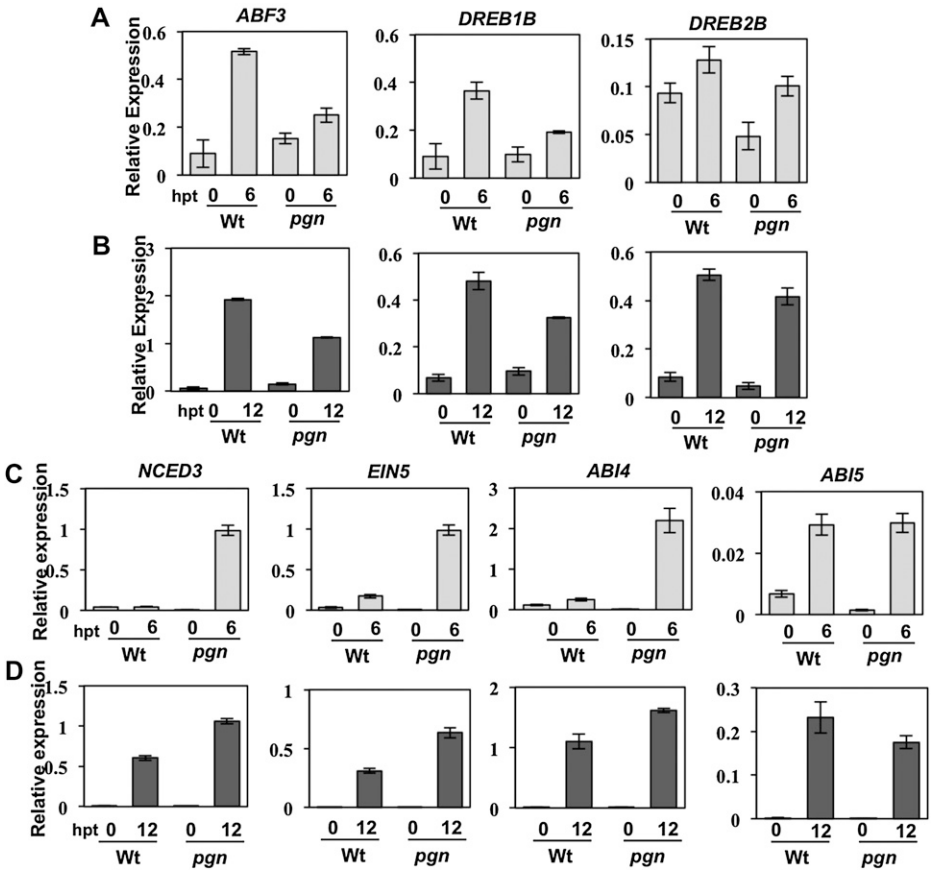

Figure 7. Expression of genes involved in ABA-related functions in the *pgn* mutant. Expression of ABA-related genes in ABA- (A and C) or NaCl-treated (B and D) seedlings. hpt, Hours posttreatment; Wt, wild type. Treatment and quantification of expression was performed as described in "Materials and Methods."

as a downstream component of the *GUN1*-regulated retrograde response pathway to photooxidative stress (Koussevitzky et al., 2007). ABI4 is a transcriptional regulator of sugar signaling required for plant responses to ABA, Glc, and salt (Finkelstein et al., 1998; Huijser et al., 2000; Quesada et al., 2000; Bossi et al., 2009). Based on the hypersensitive phenotypes and altered *ABI4* expression in the *pgn* mutant, we assayed the level of *LHCB1* transcripts in NF-treated *pgn* to determine if it plays a role in plastid-to-nucleus signaling. In response to NF, *LHCB1* was repressed in both wild-type and *pgn* seedlings without any observable difference in the level of suppression, suggesting that *PGN* does not function in photooxidative retrograde signaling (Supplemental Fig. S7).

DISCUSSION

The Arabidopsis PPRP family consists of hundreds of proteins whose function is largely unexplored (Lurin et al., 2004). In this study, we describe the biological function of *PGN*, encoding a mitochondrial PPRP, in plant stress responses. Our study provides molecular and genetic evidence for the critical function of PGN in plant responses to a subset of abiotic and biotic stresses. The *PGN* gene shows increased expression in response to necrotrophic infection, and its loss of function results in susceptibility to the fungal pathogens *A. brassicicola* and *B. cinerea*. The susceptibility of *pgn* is independent of SA-, ET-, and JA-mediated responses, and the mutant shows no altered resistance to virulent or avirulent strains of the bacterial pathogen *P. syringae*, suggesting the defense function of *PGN* is restricted to necrotrophic infection. However, the altered germination responses of the *pgn* mutant to ABA, ACC, Glc, and salt also suggest a critical role for *PGN* in abiotic stress tolerance (Gazzarrini and McCourt, 2001; Dekkers et al., 2004; Tuteja, 2007). Importantly, the *pgn* mutant accumulates increased levels of ABA and stress-induced ROS, both of which may underpin its various phenotypes. Furthermore, the expression profiles of many nuclear and mitochondrial genes, particularly those involved in retrograde and ABA signaling are altered by *pgn* mutation. These data, together with its mitochondrial localization, uniquely link PGN function to the regulation of stress-induced ABA responses and mitochondrial ROS homeostasis in Arabidopsis.

In contrast to SA, JA, and ET, which have long been known for their functions in plant defense, ABA is primarily known for its role in regulating abiotic stress responses (Fujita et al., 2006). Many mutants altered in ABA responses exhibit hypersensitivity to salt or Glc similar to that observed for *pgn* (Xiong et al., 2002; León and Sheen, 2003). More recently, ABA synthesis

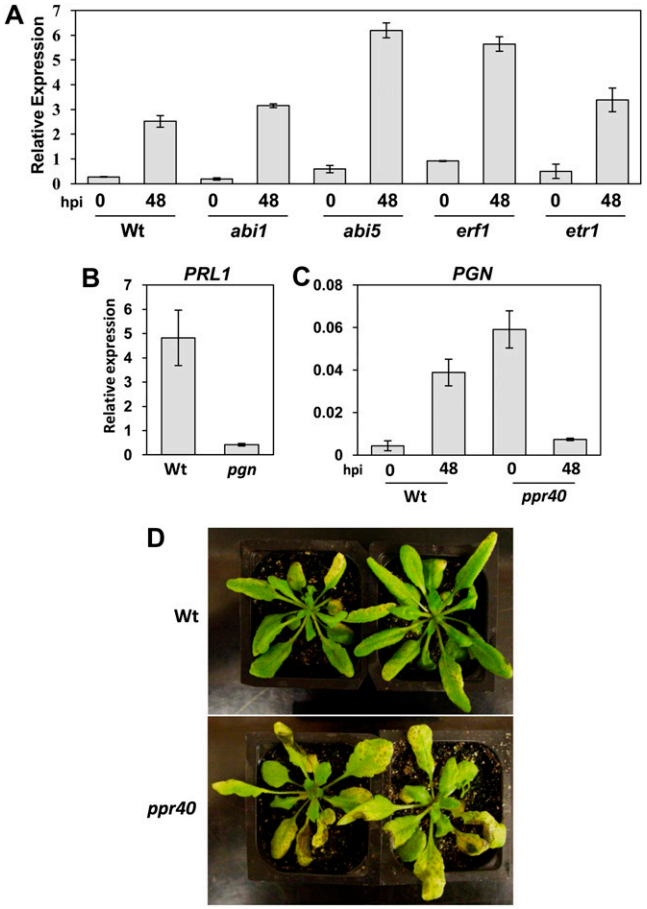

Figure 8. Impact of the ABA response pathway and the *PPR40* gene on *PGN* expression and *B. cinerea* resistance. A and C, *PGN* expression in ABA/ET response mutants (A) and the *ppr40* mutant (C) in response to *B. cinerea*. B, Basal *PRL1* expression in *pgn* and wild-type (Wt) plants. D, The *ppr40* mutant has increased susceptibility to *B. cinerea*. Images were taken 5 d after inoculation. Inoculation and quantification of gene expression were performed as described in "Materials and Methods." hpi, Hours postinoculation.

and signaling have been implicated in plant defense against pathogens (Mauch-Mani and Mauch, 2005). Although the mechanism is currently not known, PGN regulates ABA levels, which, in turn, is likely responsible for the observed mutant phenotypes including impaired disease resistance. The high levels of endogenous ABA caused by the *pgn* mutation may be due to an indirect effect of cellular perturbations, including loss of ROS homeostasis in the mitochondria or a function of the direct regulatory effect of PGN on genes involved in ABA biosynthesis. Consistent with this, in response to salt and ABA, *pgn* seedlings have markedly increased expression of the *NCED3* gene, encoding a key enzyme responsible for stress-induced ABA biosynthesis. The expression of *NCED3* directly correlates with increased ABA accumulation (Thompson et al., 2000; Iuchi et al., 2001). Thus, the germination responses of *pgn* may be a result of increased *NCED3*-mediated ABA synthesis leading to a misregulation of ABA-induced ROS generation. This notion is further supported by the ABA, salt, and sugar insensitivity conferred by *NCED3* loss of function as well as the partial alleviation of *pgn* Glc hypersensitivity by addition of ABA antagonists (Ruggiero et al., 2004; Huang et al., 2008). Alternatively, impaired ROS metabolism in the mutant may underlie its altered ABA accumulation.

ET and ABA are known to function antagonistically in a number of signaling events occurring at germination and throughout development, including the regulation of abiotic and biotic stress responses (Beaudoin et al., 2000; Ghassemian et al., 2000; Mauch-Mani and Mauch, 2005; Fujita et al., 2006). Many ABA-hypersensitive mutants have been independently isolated in genetic screens for ET insensitivity (León and Sheen, 2003). The partial restoration of *pgn* growth sensitivity to Glc by ACC is likely due to a suppression of ABA signaling and synthesis. The same can be said for the relieved growth inhibition caused by NF, an inhibitor of ABA biosynthesis (Bartels and Watson, 1978; Zeevaart and Creelman, 1988). However, *pgn* also has heightened sensitivity to ET, exhibiting an enhanced triple response characterized by decreased hypocotyl length and root elongation relative to wild-type seedlings, which is in direct contrast with the majority of published data. To our knowledge, *prl1* is the only other Arabidopsis mutation resulting in concurrent hypersensitivity to ET, ABA, and Glc (Németh et al., 1998). PRL1 is part of a spliceosome-associated complex that may contribute to regulation of alternative RNA splicing or miRNA/small interfering RNA generation (Palma et al., 2007). Interestingly, expression of *PRL1* is significantly repressed in the *pgn* mutant, suggesting that these two may have functional interaction in RNA metabolism in response to different abiotic and biotic stresses. In addition, *prl1* plants are resistant to the methylerythritol phosphate pathway inhibitor clomazone, a phenotype observed with loss of function of *LOI1*, which encodes a mitochondrial PPRP (Kobayashi et al., 2007; Flores-Pérez et al., 2010).

Though some are known to have DNA substrates, PPRPs are largely sequence-specific RNA-binding proteins associated with all stages of posttranscriptional regulation including translation (Delannoy et al., 2007). PPRPs function in RNA splicing, degradation, editing, and maturation by recruiting processing enzymes to bound transcripts (Delannoy et al., 2007; Schmitz-Linneweber and Small, 2008). Potentially, PGN may regulate stress-induced expression of transcripts involved in ABA responses or the regulation of ROS homeostasis. Alternatively, PGN may be a positive regulator of genes involved in abiotic and biotic resistance or suppress negative regulators through RNA degradation. The altered expression of both nuclear and mitochondrial genes in the mutant is consistent with a role for *PGN* in general stress tolerance rather than acting as a specific regulator of defense against pathogens. Particularly, the differen-

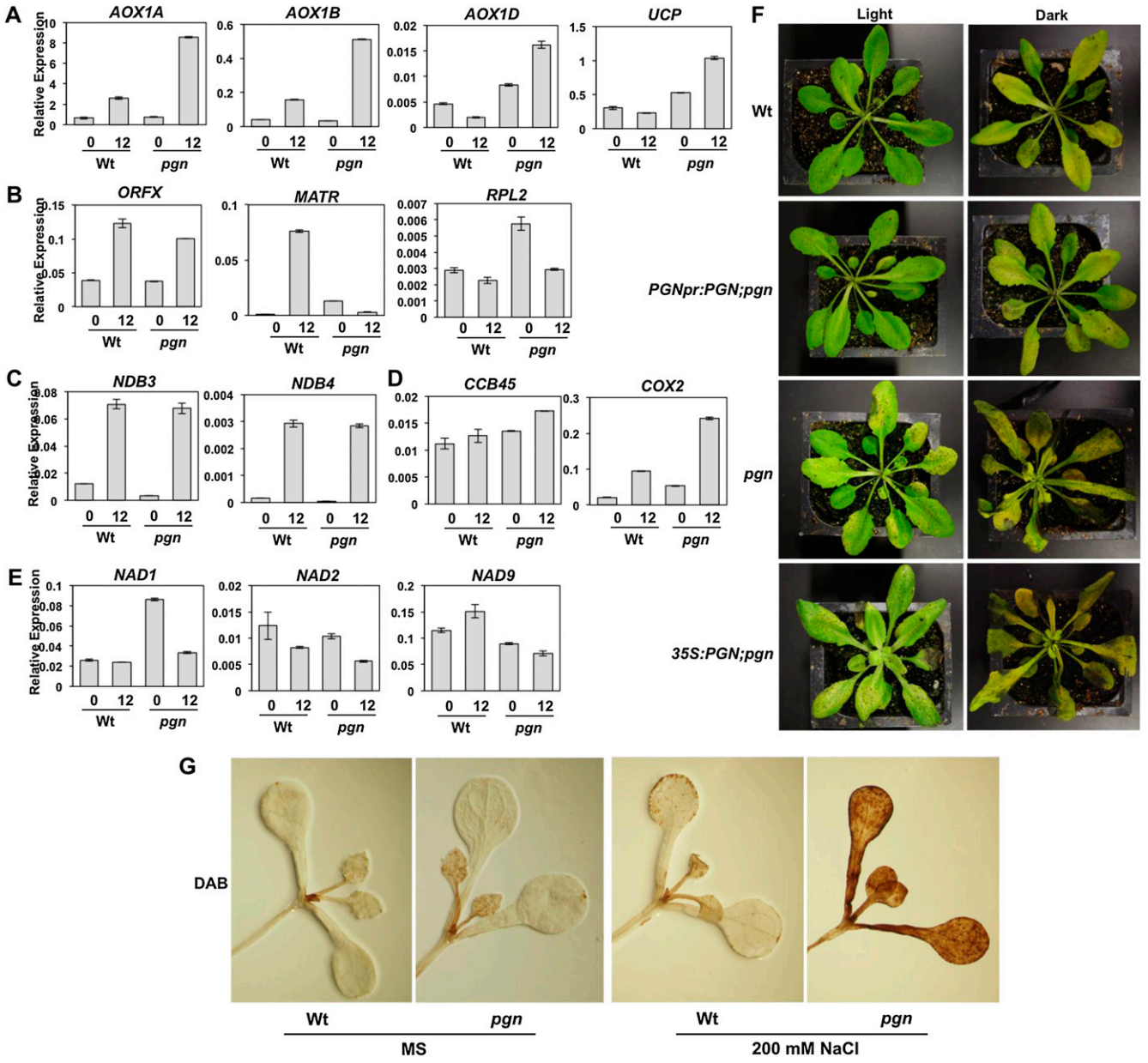

Figure 9. Expression profile of mitochondria-associated genes in the *pgn* mutant and light-dependent variations in disease resistance. A to E, Expression of genes associated with mitochondrial electron transport (A), transcription (B), oxidation/reduction (C), cytochrome *c* regulation (D), and NAD(P)H dehydrogenase subunits (E) in wild-type (Wt) and *pgn* seedlings after NaCl treatment. F, Disease responses of *B. cinerea*-inoculated plants kept in continuous dark or light conditions. Image was taken 4 d after inoculation. G, Accumulation of H_2O_2 in seedlings as revealed by DAB staining. DAB polymerizes in the presence of H_2O_2, producing a visible brown stain. Seedling treatment, plant inoculation, and quantification of gene expression were performed as described in "Materials and Methods."

tial expression of several mitochondria-encoded transcripts, including *NAD1*, *RPL2*, *NAD9*, and *MATR*, in the mutant suggests that PGN has a critical regulatory role in mitochondrial gene expression. In addition, *pgn* shows significantly higher induction of *AOX1* gene expression. *AOX* genes encode alternative oxidases that function in the maintenance of ROS levels in the mitochondria and are considered markers of mitochondrial retrograde signaling (Maxwell et al., 1999; Giraud et al., 2009).

ROS are produced not only as byproducts of cellular metabolism during electron transport but also as a result of plant exposure to abiotic and biotic stresses (Moller, 2001; Blokhina et al., 2003). Thus, the altered electron transport-associated gene expression coupled with the high level of salt-induced ROS accumulation

in the mutant strongly suggests that loss of *PGN* function results in a stress-induced misregulation of ROS metabolism. Potentially, PGN and its network of coexpressed PPRPs may coordinately affect global mitochondrial gene expression through transcript editing, thereby contributing to plant defense and maintaining cellular redox. During germination, ROS alleviate dormancy by suppressing ABA responses; however, past a threshold, they lead to oxidative stress and elicit ABA-mediated responses. Therefore, ROS generated from altered mitochondrial function may be responsible for the increased levels of ABA in *pgn* seedlings. *PPR40*, encoding a PPRP important for Complex III function in mitochondrial electron transport, was suggested to function in a similar manner (Zsigmond et al., 2008). *PPR40* was identified through a screen for ABA-hypersensitive mutants, with loss of function also resulting in decreased tolerance to salt and osmotic stress (Zsigmond et al., 2008). *ppr40* mutants generate elevated levels of ROS coincident with increased *AOX* expression (Zsigmond et al., 2008). In addition, we found *ppr40* plants are susceptible to *B. cinerea* and have significantly higher basal *PGN* expression that is repressed after infection. Based on the similarity of the phenotypes and the aberrant expression pattern of *PGN* in the *ppr40* mutant, *PPR40* may regulate *PGN* transcription.

The ACC sensitivity of the *pgn* mutant is likely also a reflection of disrupted cellular redox. Both the hypocotyls and roots of ACC-treated seedlings accumulate increased ROS in their elongation zones, thereby leading to the decreased growth characteristic of the triple response (De Cnodder et al., 2005). In plants, ROS accumulation and scavenging is largely mediated by redox events in chloroplasts and mitochondria (Noctor et al., 2007). As the triple response occurs under dark conditions, the majority of ROS production resulting from ACC treatment must occur in the mitochondria. Thus, the heighted ACC sensitivity of the mutant is likely a reflection of altered mitochondrial function rather than impaired ET signaling. This is further supported by the increased *AOX* expression, ROS accumulation, and enhanced susceptibility of the mutant to necrotrophic infection under dark conditions. Incubation in light allows inoculated plants to use chloroplasts and mitochondria in the removal of harmful ROS, thereby contributing to defense, whereas incubation in the dark necessitates relying largely only on mitochondria for ROS scavenging. This suggests the weak susceptibility of the mutant under light conditions is due to altered redox regulation in the mitochondria, with the more apparent susceptibility in the dark likely a result of loss of chloroplast contribution to ROS removal.

Its mitochondrial localization, involvement in the regulation of mitochondria-associated transcripts, and the phenotypes resulting from its loss of function all suggest a critical role for *PGN* in mitochondrial function during abiotic and biotic stress responses. Based on our data, the impaired ROS metabolism resulting from loss of *PGN* function likely leads to an up-regulation of ABA synthesis and signaling, or vice versa, accounting for the altered stress responses associated with *PGN* over- or underexpression. The relative specificity of the *pgn* mutant phenotypes, displaying altered responses only to certain pathogens and abiotic stresses, suggests the mutant is not impaired in general cell death responses due to constitutively high levels of ROS related downstream responses. However, we cannot exclude the possibility of impaired antioxidant activation in the mutant during responses to certain pathogens or stress responses. This is supported by the lack of runaway cell death in the mutant, its unaltered responses to MV, H_2O_2, and virulent *P. syringae* as well as an avirulent strain of this pathogen known to induce the hypersensitive response. Overall, the exact mechanism of function of *PGN* in plant defense and abiotic stress tolerance remains unclear. Future investigation into the molecular and biochemical basis of PGN regulation of ABA accumulation, ROS generation, target gene expression, and involvement in retrograde signaling is needed to further elucidate its functions in Arabidopsis stress responses.

MATERIALS AND METHODS

Plant Growth and Treatments

Plants were grown in soil under fluorescent light (150 μE m^{-2} s^{-1}) at 23°C ± 4°C with 60% relative humidity and a 12-h-light/12-h-dark cycle. Seeds were vernalized in water for 2 d at 4°C prior to planting. For axenic plant growth, all seeds were surface sterilized as previously described (Mengiste et al., 2003). Seeds were plated on supplemented or nonsupplemented media consisting of MS salts (PhytoTechnology Laboratories) with 2% (w/v) Suc and 0.8% (w/v) agar, pH 5.7. Transgenic seedlings were identified by selecting on MS media supplemented with appropriate antibiotics. Seed germination and seedling growth assays were also performed using MS media supplemented with different concentrations of different chemicals. All plates were vernalized at 4°C for 2 d followed by incubation at room temperature. To assay for the triple response, seeds were surface sterilized and plated on MS media containing 0 and 4 μM ACC (Sigma-Aldrich). Plates were kept in the dark and incubated 2 d at 4°C followed by 3 d at 23°C ± 2°C.

For gene expression analysis, 4-week-old soil-grown plants were sprayed with 2.5 × 104 *Botrytis cinerea* spores/mL, 5 mM SA, 1 mM MeJA, 0.5 mM ACC, or 100 μM ABA (Sigma-Aldrich). Ten-day-old seedlings grown in vitro were treated with 200 mM NaCl or 100 μM ABA supplemented liquid MS media and shaken at room temperature for 0, 6, and 12 h or 0, 3, and 6 h, respectively. Seedlings were treated with NF (Chem Service) as previously described (Koussevitzky et al., 2007). Plants were treated with H_2O_2 and MV to assay chlorophyll degradation/content as previously described (Zsigmond et al., 2008). DAB staining for H_2O_2 was performed on 10-d-old seedlings following 3 h incubation in 200 mM NaCl supplemented or unsupplemented MS media according to the manufacture's protocol (Sigmafast DAB; Sigma-Aldrich).

ABA Extraction and Quantification

Total ABA was extracted from 1 to 1.5 g of tissue from 10-d-old seedlings as previously described with the modifications of using an acetone/water/acetic acid (80%/19%/1%) solution for homogenization and (±)-3′,5′,5′,7′,7′,7′-d6 ABA as an internal standard (National Research Council Canada; Cheng et al., 2002). ABA content was then determined using liquid chromatography-tandem mass spectrometry.

RNA Extraction, Blots, and Expression Analyses

For RNA blots, total RNA was isolated as described (Lagrimini et al., 1987), separated on 1.2% agarose-formaldehyde gels, and blotted to Hybond N^+

nylon membranes (Amersham Pharmacia Biotech). Probes were labeled with ^{32}P using the random labeling system (Redi Prime II; GE Healthcare) and hybridized to blots as previously described (Church and Gilbert, 1984). Membranes were exposed to film for 24 h at −80°C (Biomax XAR Film; Kodak). Ethidium bromide staining of rRNA was used as a loading control. RNA extraction, cDNA synthesis, and quantitative reverse transcription (qRT)-PCR expression analyses were carried out as previously described (Dhawan et al., 2009). cDNA of all samples was synthesized using 3 μg DNAse-treated template RNA, AMV reverse transcriptase (Promega), and oligo(dT15) primers according to standard protocols. RT-PCR and qRT-PCR were performed using gene-specific primers with Arabidopsis (*Arabidopsis thaliana*) *Actin2* as an endogenous reference for normalization. For qRT-PCR, a minimum of three technical replicates were used for each sample with a minimum of two biological replicates. Expression levels were calculated by the comparative cycle threshold method (Applied Biosystems). Normalization to the control was performed as previously described (Bluhm and Woloshuk, 2005). Primers used are listed in Supplemental Table S1.

Fungal Cultures and Disease Assays

Fungi were cultured and maintained as described (Mengiste et al., 2003). Disease assays using *B. cinerea* strain BO5-10 and *Alternaria brassicicola* strain MUCL20297 were performed as previously described (Mengiste et al., 2003). Disease assays for virulent/avirulent *Pseudomonas syringae* pv *tomato* were done as described (Mengiste et al., 2003).

Generation of *PGN* Transgenic Lines and Mutant Identification

PGN (*AT1G56570*) overexpression lines were generated by cloning the full-length *PGN* genomic sequence behind the cauliflower mosaic virus 35S promoter in pCAMBIA99-*3XHA*. *PGN* complementation lines were generated by cloning the full-length genomic sequence of *PGN* including 1.6 kb 5′ to the start codon into pCAMBIA1380. Sequences were amplified using the KOD DNA polymerase according to the manufacturer's protocol with the addition of 1% dimethyl sulfoxide to each reaction (Novagen). Each binary vector was transferred into *Agrobacterium tumefaciens* strain GV3101 and transformed into Arabidopsis (Clough and Bent, 1998). Transgenic plants were selected on media containing hygromycin. The *pgn* (SALK_141937) mutant allele was identified from a segregating population obtained from the Arabidopsis Biological Resource Center using T-DNA and gene-specific primers (Sessions et al., 2002). Homozygous *abi1*, *abi5*, *erf1*, and *etr1* mutant alleles were also obtained from the Arabidopsis Biological Resource Center (Chang et al., 1993; Gosti et al., 1999; Finkelstein and Lynch, 2000; Berrocal-Lobo et al., 2002). Primers used are listed in Supplemental Table S1.

Construction of PGN-GFP and Visualization

The full-length genomic sequence of *PGN* was inserted into pCAMBIA99-1-*GFP* to generate a 5′ *PGN* translational fusion. The construct was verified by sequencing and introduced into *Agrobacterium* (strain GV3101), which was subsequently used for transient expression in *Nicotiana benthamiana* via agroinfiltration. Agrobacteria carrying the empty pCAMBIA99-1-*GFP* vector was used as a control. The mCHERRY mitochondrial marker was previously described (Nelson et al., 2007). Localization was observed using a Nikon Eclipse E800 epifluorescence microscope. Primers used are listed in Supplemental Table S1.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number At1g56570.

Supplemental Data

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

Supplemental Figure S1. *PGN* expression at different developmental stages.

Supplemental Figure S2. *PGN* mutation does not alter plant resistance to *P. syringae*.

Supplemental Figure S3. The *pgn* mutant is not altered in expression of defense or abiotic stress response marker genes.

Supplemental Figure S4. Multiple sequence alignment of PGN and closely related Arabidopsis proteins.

Supplemental Figure S5. Germination and growth responses of the *pgn* mutant are not altered on ACC, SA, MeJA, GA, indole-3-acetic acid, or H_2O_2.

Supplemental Figure S6. The *pgn* mutant is not impaired in oxidative stress tolerance.

Supplemental Figure S7. The *pgn* mutant is not altered in retrograde signaling from the chloroplast.

Supplemental Table S1. List of primers used in *PGN* study.

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