The Arabidopsis *BAP1* and *BAP2* Genes Are General Inhibitors of Programmed Cell Death[1][OA]

Huijun Yang, Shuhua Yang[2], Yongqing Li, and Jian Hua*

Department of Plant Biology, Cornell University, Ithaca, New York 14853

Here we identify the *BAP1* and *BAP2* genes of Arabidopsis (*Arabidopsis thaliana*) as general inhibitors of programmed cell death (PCD) across the kingdoms. These two homologous genes encode small proteins containing a calcium-dependent phospholipid-binding C2 domain. *BAP1* and its functional partner *BON1* have been shown to negatively regulate defense responses and a disease resistance gene *SNC1*. Genetic studies here reveal an overlapping function of the *BAP1* and *BAP2* genes in cell death control. The loss of *BAP2* function induces accelerated hypersensitive responses but does not compromise plant growth or confer enhanced resistance to virulent bacterial or oomycete pathogens. The loss of both *BAP1* and *BAP2* confers seedling lethality mediated by *PAD4* and *EDS1*, two regulators of cell death and defense responses. Overexpression of *BAP1* or *BAP2* with their partner *BON1* inhibits PCD induced by pathogens, the proapototic gene *BAX*, and superoxide-generating paraquat in Arabidopsis or *Nicotiana benthamiana*. Moreover, expressing *BAP1* or *BAP2* in yeast (*Saccharomyces cerevisiae*) alleviates cell death induced by hydrogen peroxide. Thus, the *BAP* genes function as general negative regulators of PCD induced by biotic and abiotic stimuli including reactive oxygen species. The dual roles of *BAP* and *BON* genes in repressing defense responses mediated by disease resistance genes and in inhibiting general PCD has implications in understanding the evolution of plant innate immunity.

Programmed cell death (PCD) is a death program actively executed by the cell. In animals, PCD is a way to sculpt tissues, maintain cell numbers, and remove unwanted or damaged cells (Jacobson et al., 1997). In plants, PCD is an integral part of plant development, occurring throughout the plant's life cycle in processes such as fertilization, xylogenesis, and senescence (Greenberg, 1996). It is also an essential component known as hypersensitive response (HR) during plant-pathogen interactions (Shirasu and Schulze-Lefert, 2000; Greenberg and Yao, 2004). HR occurs in race-specific disease resistance mediated by the host disease *R* (resistance) gene and the corresponding pathogen *avr* (avirulence) gene in an allele-specific manner (Flor, 1971). It is characterized by rapid calcium and other ion fluxes, an extracellular oxidative burst, and transcriptional reprogramming (Scheel, 1998). Plants may use an apoptotic machinery similar to those of animals and yeast (*Saccharomyces cerevisiae*) as similar morphological and biochemical features are shared for PCD in these organisms (Gilchrist, 1998; Beers and McDowell, 2001; Greenberg and Yao, 2004; Lam, 2004). Furthermore, cell death in plants is suppressed by expression of an animal antiapoptosis gene *CED9/Bcl-2* (Mitsuhara et al., 1999; Dickman et al., 2001), and an HR-like cell death is induced by the expression of animal proapoptotic genes such as *Bax* (Lacomme and Santa Cruz, 1999; Mitsuhara et al., 1999; Xu et al., 2004). However, functional equivalents of animal cell death genes have not been readily identified by sequence homology in plants and the regulation and execution of PCD in plants have yet to be understood.

PCD and disease resistance are intricately linked in plants, exemplified by the simultaneous induction of disease resistance and activation of cell death upon pathogen recognition by R proteins. A number of signaling molecules are involved in disease resistance including reactive oxygen species (ROS), salicylic acid (SA), and nitric oxide (Shirasu and Schulze-Lefert, 2000). ROS accumulate preceding cell death during HR, with biphasic oxidative bursts (Lamb and Dixon, 1997). Although ROS have been shown to trigger cell death (Van Breusegem and Dat, 2006), ROS generating NADPH oxidase complex appears to negatively regulate cell death during HR (Torres et al., 2005). SA plays a crucial molecule for systemic acquired resistance (Durrant and Dong, 2004) and it accelerates the rate of cell death in HR and amplifies a sustained oxidative burst (Shirasu and Schulze-Lefert, 2000). R proteins cloned to date largely belong to five protein families (Dangl and Jones, 2001; Martin et al., 2003). Those in the largest family in Arabidopsis (*Arabidopsis thaliana*) contain a nucleotide-binding (NB) domain and a Leu-rich repeat (LRR) domain at the carboxyl terminus, with either a coiled-coil (CC) domain or a Toll/interleukin-1 receptor

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[2] Present address: State Key Laboratory of Plant Physiology and Biochemistry, China Agricultural University, Beijing 100094, China.

* Corresponding author; e-mail jh299@cornell.edu.

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(TIR) domain at the amino terminus (Meyers et al., 2003). Although examples of direct physical interaction between Avr and R exist, emerging evidence suggests that the recognition could be indirectly mediated by other plant host proteins. In this guard hypothesis, R proteins may guard or monitor the status of the host plant proteins that are targets of pathogen Avr effector proteins (Martin et al., 2003; Chisholm et al., 2006; Jones and Dangl, 2006).

Genetic studies have identified genes required for *R* gene signaling (Dangl and Jones, 2001; Glazebrook, 2001). *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*) and *PHYTOALEXIN DEFICIENT4* (*PAD4*) are required for the function of TIR-NB-LRR proteins while *NONRACE-SPECIFIC DISEASE RESISTANCE1* (*NDR1*) is normally required for the CC-NB-LRR proteins although there are exceptions (Wiermer et al., 2005). *REQUIRED FOR MLA12 RESISTANCE* (*RAR1*), *SUPPRESSOR OF THE G2 ALLELE OF SKP1* (*SGT1*), and *HEAT SHOCK PROTEIN90* (*HSP90*) modulate R-protein accumulation and signaling competence (Azevedo et al., 2002; Schulze-Lefert, 2004; Holt et al., 2005; Azevedo et al., 2006). Intriguingly, *EDS1*, *PAD4*, and *NDR1* are implicated in the amplification of cell death and this function appears to be independent from their roles in *R*-gene-mediated defense responses (Clarke et al., 2001; Rusterucci et al., 2001). Genetic studies have also identified genes for cell death control. A number of mutants classified as lesion mimics induce spontaneous cell death that may result from defects in developmental PCD, HR control, or from necrosis and chlorosis (Shirasu and Schulze-Lefert, 2000). Some of the lesion mimic mutants have misregulation of the initiation of cell death and form small, localized, necrotic spots. More than 30 such mutants have been isolated including some of those in *accelerated cell death* (*acd*), *constitutive expressor of PR genes* (*cpr*), *lesion simulating disease* (*lsd*), and *suppressor of SA insensitivity* (*ssi*) in Arabidopsis (Lorrain et al., 2003) and *mutation-induced recessive alleles* (*mlo*) in barley (*Hordeum vulgare*; Buschges et al., 1997). About half a dozen mutants, including some *lsd* and *acd*, are unable to control the rate and extent of lesions and form chlorosis in a large area (Lorrain et al., 2003). Most of these lesion mimic mutants have altered defense responses, further indicating an intricate connection between cell death and disease resistance. Understanding how each individual gene modulates cell death is essential to deciphering cell death control and defense pathways.

The Arabidopsis *BAP1* gene is involved in defense and cell death regulation. It encodes a membrane-associated protein containing a C2 domain and has a calcium-dependent phospholipid-binding activity (Hua et al., 2001; Yang et al., 2006a). Biochemical and genetic data indicate that BAP1 is a functional partner of BON1, an evolutionarily conserved copine protein with two C2 domains at its amino terminus (Hua et al., 2001; Yang et al., 2006a). *BAP1* and *BON1* are negative regulators of defense responses. Similar to but less so than the *bon1* mutants (Hua et al., 2001; Jambunathan et al., 2001), the *bap1* loss-of-function mutants have an enhanced disease resistance to virulent pathogens and consequently dwarfed statures (Yang et al., 2006a). The defense phenotype is mediated by *SNC1/BAL*, a TIR-NB-LRR type of gene in the *RPP5* cluster (Yang and Hua, 2004; Yang et al., 2006a). Though a cognate *avr* gene has not been identified, *SNC1* is likely an *R* gene as its active mutants induce constitutive defense responses (Stokes et al., 2002; Zhang et al., 2003). The *bap1* and *bon1* phenotypes are reversed by loss-of-function mutations in *SNC1*, *EDS1*, and *PAD4* as well as by *nahG* encoding a SA-degrading enzyme (Yang and Hua, 2004; Yang et al., 2006a), indicating that *BON1* and *BAP1* are negative regulators of the *R* gene *SNC1*. The *BAP1* and *BON1* genes have additional roles other than negatively regulating *SNC1*. Overexpression of *BAP1* confers wild-type plants an enhanced susceptibility to a virulent oomycete in a *SNC1*-independent manner (Yang et al., 2006a). Furthermore, the loss of function of all *BON1* family (*BON1*, *BON2*, *BON3*) results in seedling lethality that is largely suppressed by *eds1*, *pad4*, but not by *snc1* or *nahG* (Yang et al., 2006b). Thus, *BON1* has an overlapping function with its two homologs in Arabidopsis and their shared function is not totally *SNC1* dependent.

The intriguing regulation of a NB-LRR type of *R* gene and defense responses by membrane-associated proteins BAP1 and BON1 prompted us to further investigate the function of these proteins. In this study, we molecularly and genetically characterized the *BAP1* gene and its homolog *BAP2* gene in Arabidopsis. Similarly to BAP1, BAP2 interacts with BON1 in the yeast two-hybrid system and its overexpression rescues the *bap1* phenotype. Unlike *bap1*, the *bap2* loss-of-function mutant has no apparent growth defects or increased disease resistance. However, it has an accelerated HR in response to avirulent bacterial pathogen. The *BAP1* and *BAP2* genes have overlapping functions in suppressing cell death, and the loss of both genes in Arabidopsis leads to seedling lethality that can be reverted by *pad4* or *eds1* mutations. Furthermore, overexpression of *BAP1* and *BON1* inhibits cell death induced by several *R* genes, a mouse proapototic gene *Bax*, and superoxide-generating paraquat in plants. In addition, expressing *BAP1* or *BAP2* in yeast attenuates cell death induced by hydrogen peroxide (H_2O_2). Thus the *BON* and *BAP* genes are likely general repressors of cell death and could therefore be targets of pathogen effectors and guarded by *R* genes.

RESULTS

BAP2 Is Homologous to *BAP1*

Blast search revealed a gene At2g45760 with homology to *BAP1* in Arabidopsis and we named it as *BAP2*. Using reverse transcription-PCR, we isolated a cDNA of *BAP2* and found that it encodes a small protein of 207 amino acids containing a C2 domain at the amino

terminus and a short segment at the carboxyl terminus. The deduced BAP1 and BAP2 proteins are 54% identical with homology at both the C2 domain and the C-terminal segment (Fig. 1A).

RNA-blot analysis indicates that *BAP2* is expressed at a lower level than *BAP1* (data not shown), which is consistent with the transcriptional profiling data available from The Arabidopsis Information Resource links (http://Arabidopsis.org/). *BAP2* is under a similar regulation at the transcript level as *BAP1*. Both genes are up-regulated by infections from *Botrytis cinerea*, nematode, and *Pseudomonas syringae*, treatments of chemicals ($AgNO_3$, chitin, cycloheximide, ozone, syringolin), and salt stress. They are also both up-regulated in the loss-of-function *bon1-1* mutant (referred as *bon1* from now on) and have a higher expression level at lower temperatures (Yang et al., 2006a; data not shown).

To assess the spatial expression pattern of *BAP2*, we fused the promoter of *BAP2* with the *GUS* reporter gene and generated transgenic plants carrying *pBAP2*::*GUS*. GUS staining of representative transgenic lines showed that *pBAP2*::*GUS* was ubiquitously expressed throughout the plants including leaves, stems, roots, and inflorescences, with higher activities in relatively young tissues (Fig. 1B). This pattern resembles that of *pBAP1*::*GUS* (Fig. 1B), suggesting that the *BAP1* and *BAP2* genes have similar spatial expression domains.

To determine whether *BAP2* has a similar biochemical function to *BAP1*, we expressed *BAP2* in the loss-of-function *bap1-1* mutant (referred as *bap1* from now on) under the control of the strong constitutive 35S promoter of cauliflower mosaic virus (CaMV). While *bap1* has small and curly leaves compared to the wild-type Columbia-0 (Col-0; referred as Col from now on), *p35S*::*BAP2* transgenic lines in *bap1-1* are essentially wild type in appearance (Fig. 1C), indicating that the BAP2 protein has a similar biochemical activity to BAP1.

Previous studies demonstrated that the BAP1 protein interacts with the BON1 protein in vitro and that they likely act as partners in vivo (Hua et al., 2001; Yang et al., 2006a). We asked whether BAP2 can interact with BON1 as well by using the yeast two-hybrid system (Fields and Song, 1989). BAP1 and BAP2 were each fused to the DNA-binding domain of the GAL4 transcription factor to generate GBD:BAP1 and GBD:BAP2 fusion proteins, respectively, while the A domain of BON1 was fused with the activation domain of GAL4 to generate GAD:BON1A. Coexpression of GBD:BAP2 with GAD:BON1A conferred growth to the yeast host strain on medium selecting for protein-protein interactions, similarly to that of GBD:BAP1 and GAD:BON1A (Fig. 1D), indicating a direct interaction between the BON1 and BAP2 proteins.

Because BON2 and BON3 have overlapping functions with BON1 (Yang et al., 2006b), we further determined whether BAP1 and BAP2 can interact with BON2 or BON3 in the yeast two-hybrid system. Coexpression of GBD:BAP1 or GBD:BAP2 with GAD:BON2A and GAD:BON3A, respectively, conferred yeast growth on the selection medium (Fig. 1D). It thus appears that each member of the BON family can interact with each member of the BAP family. Assessed by yeast growth, the strength of interaction differs among these protein pairs, with the weakest interaction found between BAP2 and BON2 and the strongest one found between BON1 and BAP1. These differences are yet to be validated with the analysis of expression and stability of these proteins in yeasts.

The Loss of *BAP1* and *BAP2* Function Confers Seedling Lethality

To elucidate the function of *BAP2*, we isolated a T-DNA insertion mutant of *BAP2* (SALK_052789) from the SALK collection. The T-DNA was inserted in the nucleotide sequence corresponding to Gln 67 of the encoded BAP2 protein (Fig. 1A), and no *BAP2* transcript was observed by RNA-blot analysis (data not shown). This loss-of-function mutant, named as *bap2-4* (referred as *bap2* from now on), did not exhibit any obvious growth defects, in contrast to the *bap1* mutant (Fig. 1E). However, an accelerated HR was observed in *bap2* compared to Col for *P. syringae* pv *tomato* (*Pst*) DC3000 expressing AvrRpt2. Col wild type and *bap2* were inoculated with a high concentration of *Pst* DC3000 carrying *avrRpt2*. At 8 h postinoculation (hpi), none of the Col leaves showed HR, while 50% of the *bap2* leaves already had HR at this time (Fig. 1, F and G). At 12 h, 90% of the *bap2* leaves exhibited HR while only 10% of the wild-type leaves showed HR (Fig. 1G).

To reveal possible overlapping functions between *BAP1* and *BAP2*, we attempted to generate double mutants between *bap2* and *bap1*. However, plants with the *bap1bap2* genotype could not be identified from the F2 progenies of a cross between *bap1* and *bap2*, suggesting that the homozygous mutant is either embryonic or seedling lethal. We subsequently sowed the progenies of double mutants (one heterozygous and the other homozygous) on agar plates, and found 39 out of 164 *bap1bap2*/+ and 52 out of 194 from *bap1*/+*bap2* seeds germinated but soon died at the cotyledon stage (Fig. 1H). Again, no surviving seedlings were *bap1bap2*, confirming that the double mutant is seedling lethal.

We observed dominant interactions between the *bap1* and *bap2* mutants. *bap1* is a recessive mutant with a mild growth defect (Yang et al., 2006a) and *bap2* has no obvious growth defect. However, heterozygous mutants of *bap1* and *bap2* each enhanced the phenotypes of the homozygous mutants of the other (Fig. 1I). The *bap1*/+*bap2* mutant had small and slightly curly leaves in contrast to the wild-type-looking *bap2* mutant. After bolting, its primary shoot frequently bended at the tip and died afterward. Multiple lateral shoots usually generated subsequently, giving a bushy phenotype. The *bap1bap2*/+ mutant exhibited a stronger phenotype than the *bap1* single mutant. Its leaves are very curly with water-soaked appearance, resembling those of *bon1*. The genetic interactions between *BAP1* and *BAP2* indicate that these two genes have

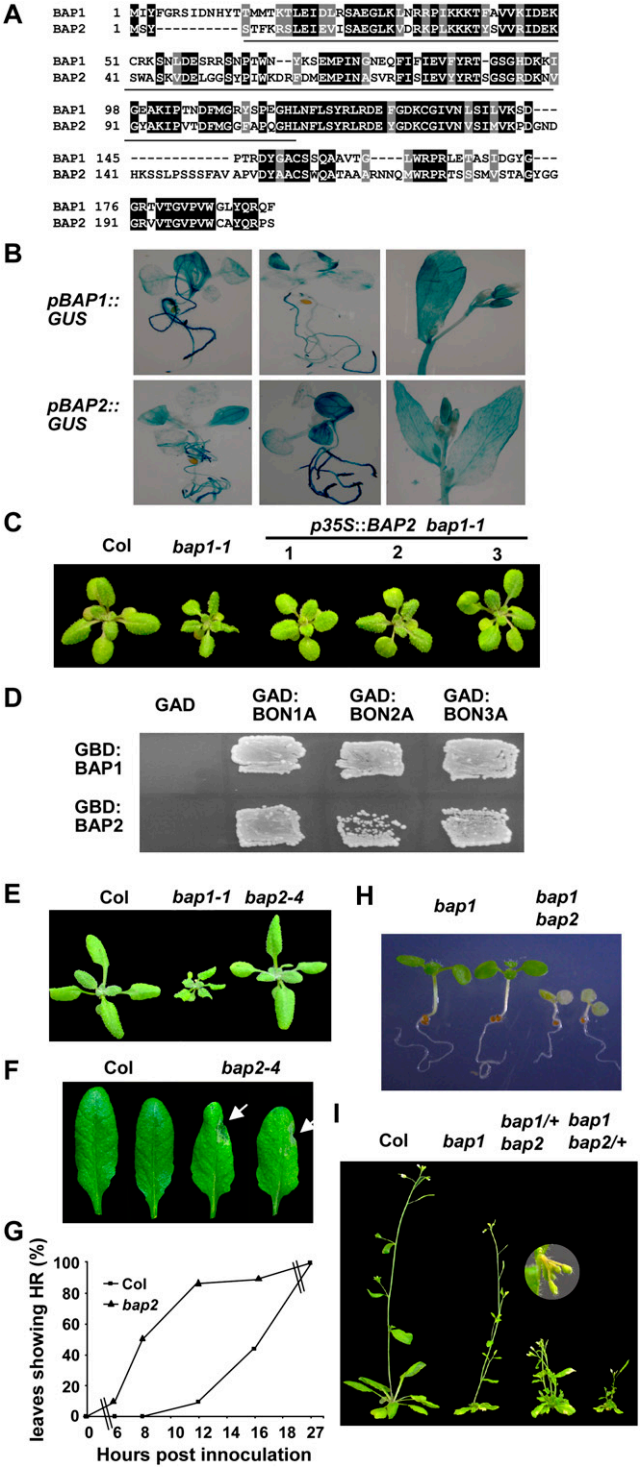

Figure 1. *BAP2* has an overlapping function with *BAP1*. A, Alignment of the amino acid sequences of BAP1 and BAP2. Identical residues are shaded in black and similar residues are shaded in gray. The C2 domains are underlined. B, Expression patterns of the *BAP1* and *BAP2* genes. Shown are representative GUS stainings of transgenic plants containing *pBAP1*::*GUS* and *pBAP2*::*GUS* at the seedling and flowering stages. Note expression in roots, young leaves, stems, and floral buds. C, *BAP2* overexpression largely rescues the *bap1-1* defect. *bap1-1* has a dwarf phenotype compared to the wild-type Col. Shown on the right are three independent transgenic lines carrying *p35S*::*BAP2* in *bap1-1*. D, BAP1 and BAP2 interact with BON1, BON2, and BON3 in the yeast two-hybrid system. GBD:BAP1 and GBD:BAP2 are fusions of BAP1 and BAP2 with the GAL4 DNA-binding domain, respectively. GAD:BON1A, GAD:BON2A, and GAD:BON3A are fusions of the A domains of BON1, BON2, and BON3 with the GAL4 activation domain, respectively. Yeast cells containing both the GAD and GBD constructs were patched on SC medium selecting for protein-protein interaction 3 d after streaking. Note the combinations of the BAP proteins with the BON proteins, but not with the GAD vector controls, grow on this medium. E, *bap2-4* has no obvious growth defects. Shown are 3-week-old seedlings of the wild-type Col, *bap1-1*, and *bap2-4*. F and G, *bap2* has an altered HR in response to *Pst* DC3000 *avrRpt2*. At 8 hpi, most of the *bap2* leaves but not the Col leaves exhibited HR indicated by white arrows (F). The percentage of leaves exhibiting HR is shown during the course of 30 h after inoculation (G). Replicated experiments yielded a similar alteration. H, The *bap1bap2* double mutant is seedling lethal. Shown are seedlings several days after germination. The two on the left are the *bap1* single mutants and the two on the right are the *bap1bap2* double mutants. I, *bap1* and *bap2* have dominant interactions. Shown are plants after bolting. *bap1bap2*/+ and *bap1*/+*bap2* have more severe phenotype than the *bap1* and the *bap2* single mutants, respectively. Insert shows a bended and yellow inflorescence stem in *bap1*/+*bap2*.

overlapping functions and that their functions are dosage dependent.

Cell Death Occurs in Mutant Combinations between *bap1* and *bap2*

We assessed cell death in different mutant combinations between *bap1* and *bap2* as their double homozygous mutant is seedling lethal. Trypan blue, a membrane impermeable reagent, was used to stain dead cells or cells with damaged cell membranes. This vital stain revealed various degrees of cell death in leaves of different mutants (Fig. 2A). None of the wild-type Col leaves (0/8) analyzed had any staining, neither did the *bap1* (0/8) or the *bap2* (0/8) single mutants. Strong staining was found in most of the leaves of *bon1-1* (9/14), consistent with previous findings (Jambunathan et al., 2001; Yang et al., 2006b). Very few leaves of *bap1*/+*bap2* (1/8) were stained by trypan blue, while most of the *bap1bap2*/+ leaves (7/12) were stained. Thus, extensive cell death occurs in leaves of *bap1bap2*/+ as in *bon1*, correlating with a severe morphological defect in leaves.

We further analyzed leaves of these mutants for autofluorescence indicative of accumulation of phenolic compounds from dead cells. No significant autofluorescence was observed in Col, *bon1*, *bap1*, *bap2*, or *bap1*/+*bap2* (Fig. 2B). In contrast, strong autofluorescence was found in *bap1bap2*/+ (Fig. 2B), indicating extensive cell death in *bap1bap2*/+.

We then asked whether the cell death phenotype in *bap1* and *bap2* mutant combinations was associated with an accumulation of ROS. To this end, we determined the relative amount of H_2O_2 in mutant plants by diaminobenzidine (DAB) that forms reddish brown precipitates when reacted with H_2O_2. Under growth conditions

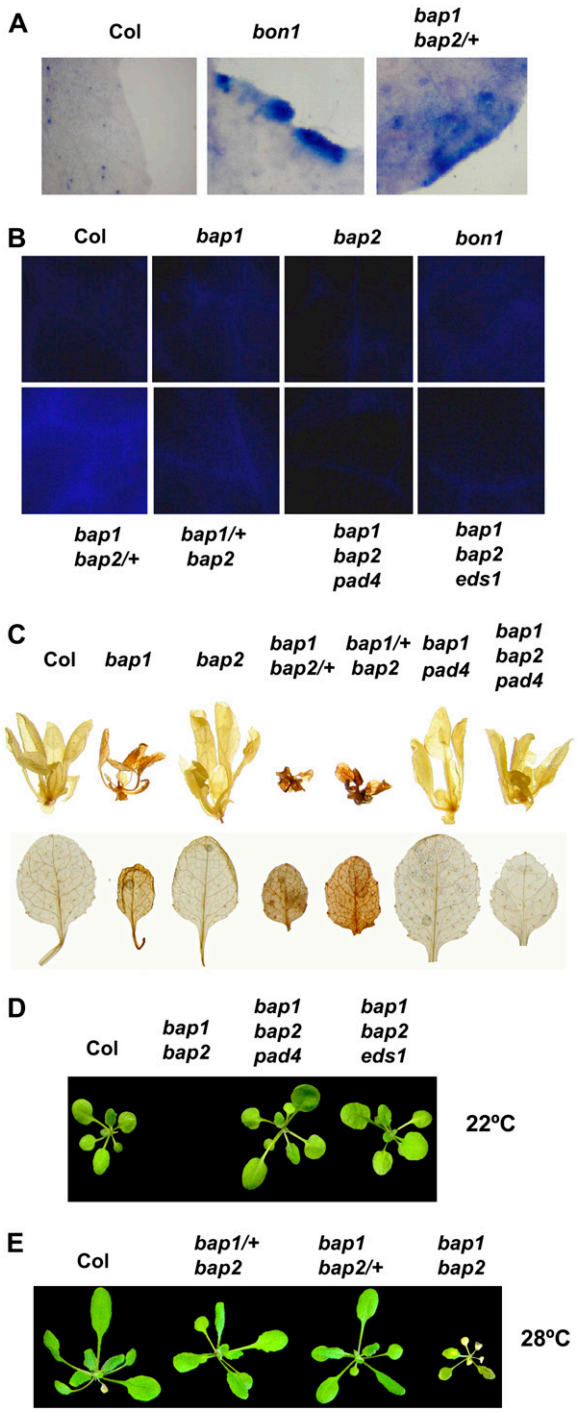

Figure 2. Cell death occurs in *bap1* and *bap2* mutant combinations. A, Trypan blue staining of representative leaves of Col, *bon1*, and *bap1bap2*/+. In contrast to wild-type Col and the *bap1* and *bap2* single mutants (data not shown), *bap1bap2*/+ has a strong trypan blue staining similar to *bon1*. B, Autofluorescence of leaves from Col, *bap1*, *bap2*, *bon1*, *bap1bap2*/+, *bap1*/+*bap2*, *bap1bap2pad4*, and *bap1bap2eds1*. *bap1bap2*/+ has the strongest autofluorescence while the *bap1* and *bap2* single mutants have no significant amount. Autofluorescence is absent in *bap1bap2pad4* and *bap1bap2eds1*. C, Accumulation of H_2O_2 in various mutants. Top section shows DAB staining of 2-week-old plants grown under constant lights and the bottom section shows DAB staining of individual leaves from plants grown under 12 h light and 12 h of darkness. Note the weak staining in *bap1*, a strong staining in *bap1bap2*/+, *bap1*/+*bap2*, but no staining in wild-type Col, *bap2*, *bap1pad4*, or *bap1bap2pad4*. D, Both *pad4* and *eds1* rescued the lethal phenotype of *bap1bap2*. Shown are 3-week-old seedlings of the wild-type Col, *bap1bap2pad4*, and *bap1bap2eds1* grown at 22°C. *bap1bap2* was dead at this stage. E, High temperature partially rescued the *bap1bap2* mutant phenotype. Shown are 3-week-old seedlings of the wild-type Col, *bap1*/+*bap2*, *bap1bap2*/+, and *bap1bap* grown at 28°C. Note *bap1bap2*/+ and *bap1*/+*bap2* are wild-type looking and *bap1bap2* is surviving but yellowing at this stage.

of both constant light and 12-h light/12-h darkness, *bap1*, but not *bap2*, had a darker staining compared to the wild-type Col. *bap1*/+*bap2* and *bap1bap2*/+ both had a stronger staining than *bap1* (Fig. 2C). Thus, H_2O_2 accumulates at a moderate level in *bap1* and at a higher level in the *bap1* and *bap2* mutant combinations.

Modulation of the *bap1bap2* Double Mutant Phenotypes by *eds1*, *pad4*, and the Environment

The lethal phenotype of *bap1bap2* could result from a heightened defense response leading to extensive cell death at very early stage of development. We assessed whether the lethal phenotype of *bap1bap2* is due to a stronger activation of *SNC1* and higher accumulation of SA in the double mutant than in the *bap1* single mutant, given that the loss-of-function mutant *snc1-11* (referred as *snc1* from now on) and the SA-degrading nahG suppressed the phenotype of *bap1*. Analysis of progenies of a *bap1bap2*/+*snc1*/+ plant and those of a *bap1bap2*/+*nahG*/+ plant indicate that neither *snc1* nor *nahG* could rescue the lethal phenotype of *bap1bap2* (data not shown).

Strikingly, the lethality of *bap1bap2* can be suppressed by mutations in *PAD4* or *EDS1*. From the F2 progenies of a cross between *bap2* and *bap1pad4* (Yang et al., 2006a), we were able to obtain *bap1bap2* plants and these plants were always *pad4* homozygous, indicating that *pad4* suppressed the lethal phenotype of *bap1bap2*. Not only was the triple mutant *bap1bap2pad4* viable, it was also wild type in appearance throughout its development (Fig. 2D). Similar rescue of lethality of *bap1bap2* was observed with the *eds1* mutation as well (Fig. 2D).

pad4 and *eds1* suppressed all other mutant phenotypes observed in the *bap1* and *bap2* mutant combinations. No autofluorescence could be seen on leaves of *bap1bap2pad4* or *bap1bap2eds1*, in contrast to the strong fluorescence on the *bap1bap2*/+ leaves (Fig. 2B). Nor was a higher level of DAB staining observed in *bap1bap2pad4*, indicating a suppression of H_2O_2 accumulation in *bap1bap2* by *pad4* (Fig. 2C).

We determined whether environmental factors can modulate the phenotypes of the *bap1* and *bap2* mutant combinations. A higher temperature of 28°C alleviates the growth defects observed in all double mutants to different degrees. Both *bap1bap2*/+ and *bap1*/+*bap2* were wild-type looking throughout the life cycle at 28°C in contrast to the dwarf phenotype at 22°C (Fig. 2E). The *bap1bap2* homozygous mutant was partially

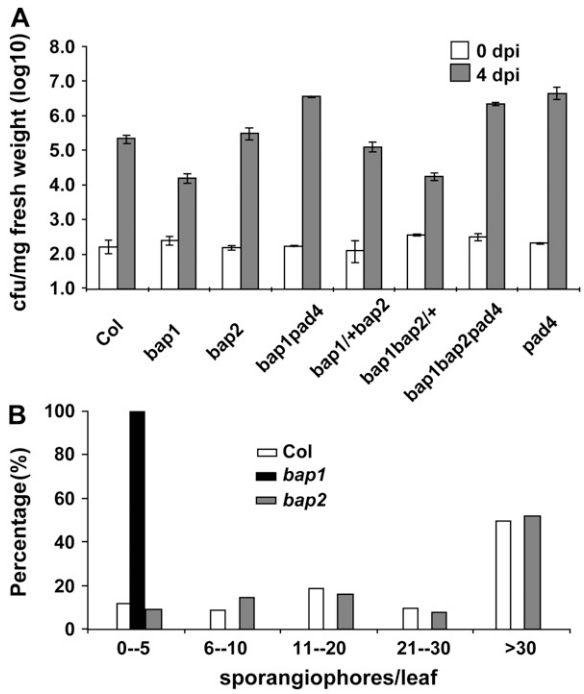

Figure 3. The *bap2* mutant does not have an enhanced disease resistance. A, *bap2* is susceptible to *Pseudomonas syringe* pv *tomato* DC3000. Plants were infected with *Pst* DC3000 and the amount of bacterial growth in the leaves was determined at 0 and 4 d post inoculation (dpi). Bacterial growth was inhibited in *bap1* but not in *bap2* compared to the wild-type Col. *bap1/+bap2* and *bap1bap2/+* had approximately the same amount of growth as *bap2* and *bap1*, respectively. *bap1bap2pad4* supports the same amount of bacterial growth as the *pad4* single mutant. B, *bap2* is susceptible to virulent growth of *Hyalopernonspora parasitica*. *H. parasitica Noco2* strain was used to infect Col, *bap1*, and *bap2*. Shown is the distribution of the number of sporangiophores per leaf formed a week later for each genotype. In contrast to *bap1, bap2* had the same amount of sporangiophore formation as the wild-type Col.

rescued by a higher growth temperature. Instead of dying immediately after germination at 22°C, the double mutant grew like the wild type at 28°C for 2 weeks after germination. However, when the wild type started bolting at approximately 3 weeks old, the double mutant turned yellow and died (Fig. 2E).

A shorter photoperiod suppressed phenotypes of some of the mutant combinations as well. *bap1/+bap2* and *bap1bap2/+* grown under a cycle of 12-h light and 12-h darkness rather than constant light were wild-type looking (data not shown). However, no *bap1bap2* could be found from progenies of *bap1/+bap2* or *bap1bap2/+* under this growth condition, indicating that the shorter photoperiod does not suppress the seedling lethality of *bap1bap2*.

The *bap2* Cell Death Phenotype Is Not Associated with Defense Responses

Because *bap1* has heightened disease resistance to virulent *P. syringae* and *Hyaloperonospora parasitica* (Yang et al., 2006a), we assessed whether *bap2* has an abnormal defense response. We challenged the *bap2* mutant with a virulent bacterial pathogen *Pst* DC3000 and found that it was as susceptible to this pathogen as the wild-type Col (Fig. 3A). Four days after infection, *Pst* grew to 4.2×10^5 colony forming units (cfu) mg^{-1} fresh weight in *bap2*, similarly to the level of 3×10^5 in the wild type, while its growth was reduced to 1.1×10^4 in *bap1*. *bap2* was also as susceptible to virulent *H. parasitica* as the wild type. While no sporangiphores were found on *bap1* a week after spray inoculation, *bap2* supported the same amount of growth of this pathogen as the wild-type Col (Fig. 3B).

Given that *bap1* and *bap2* enhanced each other's morphological and cell death phenotype in a dominant manner, we asked whether the same is true for the disease resistance phenotype. Growth of *Pst* DC3000 was analyzed in the *bap1/+bap2* and *bap1bap2/+* mutants. *Pst* propagated to 1.8×10^4 cfu mg^{-1} fresh weight in *bap1bap2/+*, comparable to the level of 1.1×10^4 in *bap1* (Fig. 3A), indicating that *bap2* does not dominantly enhance disease resistance in *bap1*. *Pst* grew to 1.3×10^5 in *bap1/+bap2*, similar though slightly lower than the level of 4.2×10^5 in *bap2* (Fig. 3A). No significant difference was observed in biological replica between *bap1/+bap2* and *bap2*. Thus, *bap1* and *bap2* do not dominantly enhance each other's disease resistance phenotype in contrast to the growth and cell death phenotype. In addition, *bap1bap2pad4* was as susceptible to *Pst* as *pad4* and *bap1pad4* (Fig. 3A), indicating that the resistance phenotype is mediated by *PAD4*.

Overexpression of *BAP* and *BON* Genes Inhibits PCD Induced by a Variety of Biotic and Abiotic Stimuli in Plants

The loss-of-function phenotypes indicate that the *BAP* genes are negative regulators of cell death. To determine whether they have a direct role in suppressing cell death, we analyzed their overexpression effect on PCD. First we assayed HR induced by *Pst* DC3000 carrying avirulent effectors in Arabidopsis. Wild-type Col plants were infiltrated with *Pst* DC3000 (*avrRpt2*) together with *Agrobacterium* containing *p35S::BON1*, *p35S::BAP1*, or an empty vector. At 14 hpi, a strong HR indicated by the collapse of tissues appeared on all leaves inoculated with *Pst* DC3000 (*avrRpt2*) together with the vector control (Fig. 4A). Agroinfiltrations with *p35S::BAP1* or *p35S::BON1* did not affect HR when compared to the vector control, although they occasionally slightly delayed its onset. In contrast, HR was not observed at 14 hpi when *p35S::BAP1* and *p35S::BON1* were simultaneously agroinfiltrated (Fig. 4A), and it only started to develop at approximately 18 hpi, indicating that *BAP1* and *BON1* together inhibited HR induced by avrRpt2.

We additionally tested the effect of *BAP1* and *BON1* overexpression on HR induced by another avirulent strain *Pst* DC3000 (*avrRpm1*). At 5 to 6 hpi, a strong HR was induced by *avrRpm1* when agroinfiltrated with the vector control. Agroinfiltration with *p35S::*

BAP1 or *p35S::BON1* alone did not significantly affect the development of HR. However, HR was not observed until 8 to 9 hpi with simultaneous agroinfiltration of *BAP1* and *BON1* (Fig. 4A). The suppression for both avirulent strains was consistently seen in replicated experiments. Therefore, overexpression of *BON1* and *BAP1* together in Arabidopsis greatly delayed HR induced by avirulent bacterial pathogen *Pst* DC3000 with two different effector proteins.

We subsequently analyzed the effect of overexpression of *BAP1* and *BON1* on PCD induced by other R proteins. Transient coexpression of a potato (*Solanum tuberosum*) NB-LRR type of R protein Rx and its elicitor PVX coat protein (CP) was shown to induce HR in *Nicotiana benthamiana* (Bendahmane et al., 2000). A collapse of cells indicative of HR was observed in leaf area agroinfiltrated with Rx and CP at 36 hpi. Coagroinfiltration with the vector alone did not alter the onset or the progression of HR. However, when *p35S::BAP1* or *p35S::BON1* were coagroinfiltrated, HR was either suppressed or greatly reduced at 36 hpi (Fig. 4B). In some repeats, no HR was ever developed over the following 5 d observation. Coagroinfiltration of *p35S::BAP1* and *p35S::BON1* together did not appear to have a stronger effect in HR suppression.

Given that *BAP1* and *BON1* inhibit HR induced by R proteins, we further tested whether the *BAP1* and *BON1* genes can suppress PCD induced by reagents other than R proteins in plants. The mouse *Bax* gene belongs to the apoptotic *Bcl-2* family and is shown to induce cell death response in plants resembling HR (Lacomme and Santa Cruz, 1999; Kawai-Yamada et al., 2001; Abramovitch et al., 2003). We infiltrated leaves of *N. benthamiana* with *Agrobacterium* containing the *Bax* gene under the control of a dexamethasone (DEX) inducible promoter (*pDEX::Bax*; Kawai-Yamada et al., 2001) and induced *Bax* expression by spraying the inoculated leaves with DEX. Cell death occurred at 72 hpi, manifested by a transparent and collapsed infiltrated area (Fig. 4C). Coagroinfiltration with either *p35S::BAP1* or *p35S::BON1* did not consistently affect the rate or extent of cell death compared to the vector control. *p35S::BAP2*, however, sometimes inhibited *Bax*-induced cell death at 72 hpi (Fig. 4C). Strikingly, when *p35S::BAP1* and *p35S::BON1* were simultaneously agroinfiltrated with *pDEX::Bax*, no obvious cell death was observed at 72 hpi when the control areas exhibited strong cell death (Fig. 4C). Similar suppression of cell death was observed when *p35S::BAP2* and *p35S::BON1* were coagroinfiltrated. In both cases, cell collapse started at 90 hpi and occurred to a full extend at 114 hpi in *BON1* and *BAP1/BAP2* coinfiltrated areas. Therefore, *Bax*-induced cell death was delayed by 1 to 2 d with overexpression of *BON1* together with *BAP1* or *BAP2*.

BAP1 and *BAP2* Inhibit Cell Death Induced by ROS in Arabidopsis and Yeast

The *BAP* transcripts are induced by a number of biotic and abiotic stimuli and the common feature of these treatments is probably ROS. Considering that they are capable of inhibiting PCD, we asked whether overexpression of the *BAP* genes can inhibit cell death induced by ROS. To this end, we compared Col Arabidopsis lines containing the *35S::BAP1* transgene (Yang et al., 2006a) to the wild-type Col in paraquat sensitivity. Paraquat is a redox-active compound that generates superoxide anion in the cell, causing cell damage and cell death (Tsang et al., 1991). We found that overexpression of the *BAP1* gene protects cells from these damages. Wild-type leaf discs treated with paraquat had chlorophyll loss and cholorosis over 2 d, while leaf discs of *35S::BAP1* transgenic lines stayed green under the same treatment (Fig. 4D), indicating a protective role of *BAP1* against ROS.

We further asked whether the *BAP* genes can protect nonplant species from ROS-induced cell death. We expressed the *BAP1* and *BAP2* genes under the control of the constitutive ADH promoter in yeast, and assayed their effects on cell death induced by ROS. Yeast cells were treated with 10 mM of H_2O_2 to induce PCD and cell survival rates were counted 12 h after the treatment. Only 1% of cells containing an empty vector survived the H_2O_2 treatment compared to the mock treatment (Fig. 4E). In contrast, cells expressing either *BAP1* or *BAP2* had significantly higher survival rates (Fig. 4E). A total of 2.6% and 4.9% of cells survived for two independent *BAP1*-expressing strains, respectively, while 18.2% and 20.4% of cells survived for two independent *BAP2*-expressing strains, respectively. The increase in survival rates by expressing *BAP1* and more so by *BAP2* was observed in repeated experiments treated with 10 mM of H_2O_2 as well as in similar experiments treated with 5 mM of H_2O_2 (data not shown).

DISCUSSION

Interaction among the *BAP* and *BON* Genes

In this study, we characterized the function of *BAP1* and *BAP2*, two homologous genes encoding small C2 domain-containing proteins. In contrast to the single *bap1* mutants that exhibit a constitutive defense response phenotype, the *bap2* single mutant does not show any obvious growth defects or enhanced disease resistance. *bap2* did exhibit an accelerated HR to an avirulent *Pseudomonas* strain, suggesting that *BAP2* has a role in modulating PCD. Furthermore, the *bap1bap2* double mutant is seedling lethal and the heterozygous mutant of one gene can enhance the homozygous mutant of the other. These genetic interactions indicate that *BAP1* and *BAP2* have unequal redundancy with *BAP1* playing a major role. They also indicate that the amount of activities conferred by *BAP1* and *BAP2* are critical for the process they regulate. This activity decreases roughly in the order of *BAP1BAP2*, *BAP1bap2/+*, *BAP1bap2*, *bap1/+BAP2*, *bap1/+bap2/+*, *bap1/+bap2*, *bap1BAP2*, *bap1bap2/+*, and *bap1bap2*, and it correlates

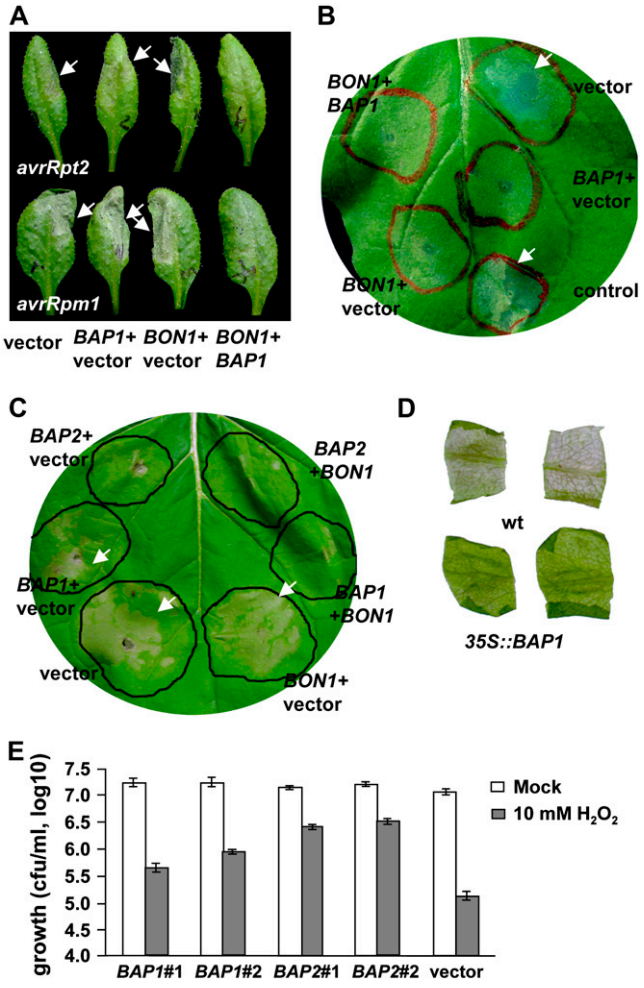

Figure 4. Overexpression of *BAP1* and *BAP2* suppresses PCD. A, BAP1 and BON1 together suppress HR triggered by *Pst* DC3000 harboring *avrRpt2* and *avrRpm1*. DC3000 strains were inoculated on Arabidopsis leaves to induce HR indicated by the collapse of cells (marked by white arrows). *p35S*::*BAP1*, *p35S*::*BON1*, or an empty vector were agroinfiltrated together with DC3000. Shown are leaves 8 hpi for *avrRpm1* and 17 hpi for *avrRpt2*. Combination of *p35S*::*BAP1* and *p35S*::*BON1* significantly inhibits HR induced by both *AvrRpt2* and *AvrRpm1*. *p35S*::*BAP1* appears to have a weak suppression of HR induced by *AvrRpt2*, but it was not consistently observed. B, Both *BON1* and *BAP1* inhibit HR induced by the R protein Rx in *N. benthamiana*. *Rx* and its effector *CP* were agroinfiltrated on leaves (marked by red circles) to induce HR. Except for the control area, all other areas were coagroinfiltrated with *BON1*, *BAP1*, or the empty vector singly or combined with the same total amount of *Agrobacterium* cells in each infiltrated area. Shown is a representative leaf at 60 hpi. Note the cell collapse in the vector and the control areas (indicated by white arrows). C, *BAP1* and *BAP2* suppress *Bax*-induced cell death in *N. benthamiana*. Leaf areas marked by black circles were agroinfiltrated with *pDEX*::*Bax* and *Bax* expression was induced by spraying the whole leaf with DEX. These areas were also agroinfiltrated with *BAP1*, *BAP2*, *BON1*, and the empty vector either singly or combined with the same total amount of agrobacteria for each area. Shown is a representative leaf at 72 hpi. Cell death occurred in area coinfiltrated with the vector control, *BON1*, and *BAP1* singly (indicated by white arrows). Cell death is slightly suppressed by *BAP2* and is greatly suppressed by coexpression of *BAP1 or BAP2* together with *BON1*. D, *BAP1* overexpression confers paraquat resistance. Leaf discs from the wild-type Col and *35S*::*BAP1* transgenic plants were floated on paraquat solution (4 μM) for 2 d before pictures were taken. E, Yeast strains transformed with *BAP1*, *BAP2*, or the empty vector pAD4M were treated with 10 mM of H_2O_2 or water (mock). Shown is the amount of live cells 12 h after treatment in two *BAP1*, two *BAP2*, and one vector transformants from three replicates. *BAP1* and especially *BAP2* greatly increased the survival rates of yeast cells treated with H_2O_2.

with an increase in morphological phenotypic severity from wild type to lethality. Considering that expressing *BAP2* under the CaMV 35S promoter rescued the *bap1* single mutant phenotype, we suspect that the *BAP2* might have a lower biological activity than *BAP1* in terms of the protein amount, protein expression domain, and/or protein activity.

Molecular genetic analysis in this study supports the previous model that BAP1 is a functional partner of BON1, and it further indicates that the BAP proteins are functional partners of the BON proteins. Overexpressing *BAP1* and *BON1* together but not singly inhibits HR induced by avirulent *Pst* and cell death induced by *Bax*, indicating that the BAP1 and BON1 proteins work together to modulate cell death. In addition, the loss of the *BAP* family function results in seedling lethality similarly to the loss of the *BON* family function, and the lethality can both be suppressed by *eds1* and *pad4*. Thus the *BAP1* family and the *BON1* family carry similar functions. Nevertheless, the suppression by *eds1* and *pad4* is more complete for *bap1bap2* than for *bon1bon2bon3*, suggesting that the *BON* genes might play a greater role than the *BAP* genes in Arabidopsis.

The fact that BAP1 and BON1 proteins could interact with each other raises the question whether there are specific pairs of interaction between the BON proteins and the BAP proteins. The yeast two-hybrid assay demonstrated that each protein of the BON family can interact with each member of the BAP family although some interactions appear to be stronger than others. This suggests that in plants there could be multiple interactions between the BON and BAP proteins. This hypothesis is supported by the observation that the *bon1bap1* double mutant has a stronger phenotype than the *bon1* or *bap1* single mutants (H. Yang and J. Hua, unpublished data). Thus BAP1 and possibly BAP2 associate in a functional manner with BON2 or BON3 in addition to the BON1 protein in plants. In addition, promoter-GUS analyses of the *BON1* family and the *BAP1* family indicate some overlapping expression domains of these genes (Yang et al., 2006b). Therefore, multiple protein complexes might form between BON and BAP proteins to provide robustness and/or specificities to the system.

Regulation of Cell Death and Defense by the BAP and BON Proteins

In this study, we identified a more direct role of the *BAP* family in the control of PCD across the kingdoms.

The loss of function of some of the *BAP* and *BON* genes (singly or in combination) leads to microlesions, accelerated HR, or lethality, implicating them as negative regulators of cell death. Quite a few negative regulators of cell death have been identified based on the phenotype of lesions induced by their loss-of-function mutants. However, the regulation could formally be indirect as some lesion mimic mutants are shown to result from the perturbation of metabolic pathways (Mittler et al., 1995; Molina et al., 1999). *rin4*, *bon1*, and *bap1* are the few known to result from activation of specific *R* genes (Axtell and Staskawicz, 2003; Mackey et al., 2003; Yang and Hua, 2004; Yang et al., 2006a). It is thought that plant host genes such as *RIN4* are targeted by plant pathogens and are subsequently monitored or guarded by *R* genes (Jones and Dangl, 2006). Some other cell death regulators such as *MLO*, though not implicated in specific *R* gene regulation, might also be targeted and manipulated by pathogens (Panstruga, 2005). Understanding the cellular function of these host target genes is of great interest in light of the evolution of plant innate immunity. Here we found a direct role of *BAP* and *BON* genes in inhibiting PCD by showing that PCD induced by a variety of reagents can be inhibited by overexpression of *BAP* and *BON* genes in different species across the kingdoms. These include HR induced by bacterial effector proteins AvrRpt2 and AvrRpm1 in Arabidopsis, HR induced by the R protein Rx in *N. benthamiana*, PCD induced by a mammalian apotopic *Bax* gene, and ROS-generating chemical paraquat. More strikingly, cell death in yeast induced by H_2O_2 is inhibited by *BAP1* and *BAP2*. The effect of overexpression on diverse PCD indicates that the *BON* and *BAP* genes may modify a common component of PCD shared by different organisms. The *BAP* genes may act downstream of the production of H_2O_2 in PCD, indicated by their suppression of H_2O_2-induced cell death in yeast. It is supported by the observation that overexpression of *BAP1* and *BON1*, though inhibits HR, did not appear to alter the onset of H_2O_2 production during *Bax*-induced cell death (Y. Li and J. Hua, unpublished data).

Direct regulators and executors of PCD in plants have also been identified by their PCD suppressing activity when they are overexpressed in animals, yeasts, and plants. These include an endoplasmic reticulum-associated BAX INHIBITOR-1 (BI-1; Kawai-Yamada et al., 2001; Watanabe and Lam, 2006; Ihara-Ohori et al., 2007), a transcription factor AtEBP (Pan et al., 2001; Ogawa et al., 2005), a vesicle-associated protein VAMP (Levine et al., 2001), and an AGC kinase Adi3 (Devarenne et al., 2006). These proteins possess a diverse variety of biochemical activities and localize to different cellular compartments, suggesting the involvement of many biochemical and cellular processes in regulating or executing PCD. The BAP1 and BON1 proteins are membrane associated and they possess a calcium-dependent phospholipid-binding activity. The BAP and BON proteins could be potentially functionally connected with AtBI-1 that was shown to interact with calmodulin and maintain calcium homeostasis. They might also work closely with VAMP as C2 proteins often play a role in membrane trafficking. Further investigation of the inhibitory activity of cell death by BON1 and BAP1 should generate insights into regulation of PCD in plants.

The *BAP* and *BON* genes appear to be unique among these direct repressors of PCD in that they are implicated in regulating specific NB-LRR type of *R*-like genes as well. Their loss-of-function mutants exhibit enhanced disease resistance to a variety of virulent pathogens through activating *R* genes. For instance, the loss of *BON1* function leads to enhanced resistance via activating an accession-specific TIR-NB-LRR gene *SNC1* (Yang and Hua, 2004), indicating that the BON1 protein could be monitored (guarded) by the *R SNC1* gene. No other genes with a direct PCD suppressing activity when overexpressed have yet been identified as being monitored by specific *R* genes. Overexpression of *BI-1* from barley weakened resistance conferred by the *mlo* mutation and an *R* gene *MLA12* to a fungal pathogen *Blumeria graminis* (Eichmann et al., 2006). This is likely due to its general effect on H_2O_2 burst and it is yet to be determined whether or not the loss of *BI-1* function will specifically trigger the activation of specific *R* genes like *MLA12*.

The dual function of *BAP* and *BON* genes in cell death and defense responses, similarly observed in *MLO* and *lsd* among others, probably reflects an intrinsic relationship between these two processes as exemplified by HR being an integral part of most of the *R*-mediated disease resistance. We favor the model that the *BAP* and *BON* genes have an ancient role in cell death control and an evolved role in plant defense response. This is consistent with the *BON* genes as members of the copine gene family found not only in plants but also in animals. It is unclear whether or not the *BAP* genes are evolutionarily conserved because the most significant signature of their encoded proteins is the C2 domain that is widely present in many signaling molecules. The striking feature of *BAP1* is its extreme responsiveness to numerous biotic and abiotic stimuli ranging from singlet oxygen species, temperature variation, wounding from bacterial infection, to even butterfly egg oviposition (op den Camp et al., 2003; Yang et al., 2006a; Little et al., 2007). *BAP2* and *BON1* respond to at least some of these stimuli but apparently to a lesser degree. The responsiveness to diverse stimuli suggests that the *BAP* and *BON* genes may serve as signaling molecules or maintain calcium or lipid homeostasis in stress responses, and the loss of these activities results in cell death. The suppression of the *bap* and *bon* phenotypes by *eds1* or *pad4* indicates that *BAP* and *BON* genes regulate a cell death pathway mediated by *EDS1* and *PAD4*. Emerging evidence has strongly implicated *EDS1* and *PAD4* in transducing redox signals (Mateo et al., 2004; Ochsenbein et al., 2006). It is tempting to speculate that the *BAP* and *BON* genes are responsive to ROS and/or calcium signals and modulate ROS signaling in stress responses.

The BAP and BON molecules might become targets of pathogen effector proteins because of their ancestral role in cell death control during the evolution of plant innate immune system (Jones and Dangl, 2006). Indeed, the *bon1* and *bap1* mutants have heightened defense responses that are at least partially mediated by a TIR-NB-LRR type of *R* gene *SNC1*. It is possible that the loss of the BON1 or BAP1 proteins is interpreted by plants as the result of the invasion of a pathogen and thus triggers the activation of R proteins to mount defense responses. Multiple *R* genes in addition to *SNC1* are likely regulated by the *BON* family and the *BAP* family, as the *bon1bon2*, *bon1bon3*, and *bap1bp2* double mutants have stronger phenotypes independent of *SNC1* than the *bon1* or *bap1* single mutants. In addition, the *bon* or *bap* mutant combinations exhibit phenotypic variations in different accession backgrounds (Yang et al., 2006b; J. Hua, unpublished data), suggesting the involvement of multiple accession-specific *R* genes. It has yet to be determined whether the regulation of BON and BAP proteins on R proteins is similar to that of RIN4 on RPM1 and RPS2. Current data do not distinguish models of regulation at the protein level or the RNA transcript level. Future studies on the general PCD inhibitor *BAP* and *BON* genes should shed light not only on the regulation of defense responses in plants but also PCD in other kingdoms.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown at 22°C or 28°C under continuous fluorescent light (100 μmol m^{-2} s^{-1}) with 50% to 70% relative humidity unless specified otherwise. Arabidopsis seeds were either directly sowed on soil or selected on plates before being transferred to soil. For bacterial pathogen tests, plants were grown at 22°C under a photoperiod of 12 h of light for 2 weeks (for dipping inoculation) or 1 month (for infiltration inoculation).

The *bap2-4* mutant was isolated from the Salk T-DNA collection (http://signal.salk.edu/cgi-bin/tdnaexpress). The T-DNA insertion site was confirmed by sequencing PCR products amplified from the mutant with T-DNA primers and gene-specific primers.

Yeast Two-Hybrid Analysis

BAP1 and BAP2 were each fused with the DNA-binding domain of the GAL4 transcription factor in the yeast (*Saccharomyces cerevisiae*) vector pGBD-C2 with a Trp auxotroph marker (James et al., 1996). The A domains of BON1, BON2, and BON3 were each fused with the activation domain of GAL4 in the yeast vector pGAD-C2 with a Leu auxotroph maker (James et al., 1996). pGBD:BAP1 and pGBD:BAP2 were each cotransformed with pGAD:BON1A, pGAD:BON2A, and pGAD:BON3A, respectively, into the yeast strain PJ69-4 (James et al., 1996). Transformants with both the GBD and GAD constructs were selected on synthetic complete (SC) medium without Trp and Leu. Protein interactions were assayed by growing the transformants on SC medium without adenine, His, Trp, and Leu.

RNA-Blot Analysis

Total RNAs were extracted from 3-week-old plants using TriReagents (Molecular Research) according to the manufacturer's protocol. Twenty micrograms of RNA for each sample were resolved on 1.2% agarose gels containing 1.8% formaldehyde. Ethidium bromide was used to visualize the rRNA bands to ensure equal loading. RNA gel blots were hybridized with gene-specific, ^{32}P-labeled, single-stranded DNA probes.

Pathogen Resistance Assay

Bacterial growth in Arabidopsis was monitored as described with some modifications (Tornero and Dangl, 2001). *Pseudomonas syringae* pv *tomato* DC3000 was grown overnight on the Kapadnis-Baseri medium and resuspended at 10^8 cfu mL^{-1} in a solution of 10 mM $MgCl_2$ and 0.02% Silwet L-77. Two-week-old seedlings were dip inoculated with bacteria and kept covered for 1 h. The amount of bacteria in plants was analyzed at 1 h after dipping (day 0) and 4 d after dipping (day 4). The aerial parts of three inoculated seedlings were pooled for each sample and three samples were collected for each genotype at one time point. Seedlings were ground in 1 mL of 10 mM of $MgCl_2$ and serial dilutions of the ground tissue were used to determine the number of cfu per gram of leaf tissues.

For HR test, *Pst* DC3000 with avirulent genes were resuspended at 10^8 cfu mL^{-1} and infiltrated into leaves of 4-week-old Arabidopsis plants. Infiltrated leaves were monitored hourly for symptoms of cell collapse.

Hyaloparanospora parasitica Noco2 strain was propagated on the Col accession of Arabidopsis. Conidiospores were suspended in water at a concentration of 40,000 spores per mL and spray inoculated onto 2-week-old plants that were subsequently kept covered at 16°C. The number of sporangiophores formed on the first two true leaves was counted a week later. Approximately 100 leaves were counted for each genotype.

Agrobacterium-Mediated Transient Expression

Genes to be expressed are cloned into binary vectors and transformed into *Agrobacterium tumefaciens* strain C58C1 containing the virulence plasmid pCH32 (Rairdan and Moffett, 2006). *Agrobacterium* infiltrations were performed as described (Bendahmane et al., 2000) with modified inoculation concentrations as specified.

The genomic fragments of the *BAP1*, *BAP2*, and *BON1* genes were expressed with the CaMV 35S promoter in the binary pGreen0229 vector (http://www.pgreen.ac.uk/). Agrobacterial cells containing *BON1*, *BAP1*, *BAP2*, or the empty vector were each resuspended in the infiltration buffer (10 mM $MgCl_2$, 10 mM MES, and 150 μM Acetosyringone) at 0.5 OD_{600}. Cells with Rx or CP were resuspended at 0.2 OD_{600} and combined at 1:1 to make the Rx and CP mixture. Cells containing the *pDEX:Bax* were resuspended at 0.5 OD_{600} 2 h prior to infiltration. A total of 50 μM of DEX was sprayed onto *Nicotiana benthamiana* leaves 15 h after infiltration.

Cell Death Analysis in Plants

Autofluorescence of leaf tissues was examined as described (Adam and Somerville, 1996). Trypan blue staining was performed as described (Bowling et al., 1997). DAB was dissolved in 50 mM of Tris-acetate (pH 5.0) at a concentration of 1 mg/mL. Leaf discs or whole seedlings were punched out, placed in the DAB solution, and vacuum infiltrated till the tissues were soaked. They were then incubated at room temperature in the dark for 24 h before the tissues were cleared in boiling ethanol (95%) for 10 min.

For paraquat treatment, leaf discs from 3-week-old plants were floated on 4 μM of paraquat. They were first kept in dark for 1 h and then incubated under light for 2 to 3 d.

Cell Death Test in Yeasts

The coding regions of the *BAP1* and *BAP2* genes were cloned into the pAD4M vector under the control of the ADH promoter (from Dr. G. Fink). Constructs were transformed into yeast strain PJ69-4 by LiAc-mediated transformation (http://mgwww.mbi.ucla.edu/node/124). Two independent transformants of *BAP1* and *BAP2* were used for cell death test. Yeast cells were grown in selective liquid medium (SC-Leu) for 36 h, collected by centrifugation, washed three times with water, and resuspended in fresh medium at a concentration of 0.5 OD_{600}. Each sample was split into two halves with one treated with H_2O_2 at a final concentration of 10 mM or 5 mM and the other with water as mock control. The amount of live cells at 12 h after treatment was analyzed by growing serial dilutions onto rich media.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_130139.

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