BAP1 and BAP2 inhibit programmed cell death

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The Arabidopsis BAP1 and BAP2 genes are general inhibitors of programmed cell death.

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Abstract

Here we identify the BAP1 and BAP2 genes of Arabidopsis thaliana as general inhibitors of programmed cell death across the kingdoms. These two homologous genes encode small proteins containing a calcium-dependent phospholipid binding C2 domain. BAPI 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 BAPI 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 BAPI and BAP2 confers seedling lethality mediated by PAD4 and EDS1, two regulators of cell death and defense responses. Overexpression of BAPI or BAP2 with their partner BON1 inhibits programmed cell death induced by pathogens, the proapoptotic gene BAX and superoxide-generating paraquat in Arabidopsis or Nicotiana benthamiana. Moreover, expressing BAPI or BAP2 in yeast alleviates cell death induced by hydrogen peroxide. Thus, the BAP genes function as general negative regulators of programmed cell death 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 programmed cell death has implications in understanding the evolution of plant innate immunity.
Introduction

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 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 resistance (R) gene and the corresponding pathogen avirulence (avr) 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 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 anti-apoptosis gene CED9/Bcl-2 (Mitsuohara et al., 1999; Dickman et al., 2001), and an HR-like cell death is induced by the expression of animal pro-apoptotic genes such as Bax (Lacomme and Santa Cruz, 1999; Mitsuohara 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 (NO) (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* contain a nucleotide binding (NB) domain and a leucine rich repeats (LRR) domain at the carboxyl terminus, with either a Coiled-coil (CC) domain or a Toll/Interleukin-1-Receptor (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). *EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1)* and *PAD4 (PHYTOALEXIN DEFICIENT 4)* are required for the function of TIR-NB-LRR proteins while *NDR1 (NONRACE-SPECIFIC DISEASE RESISTANCE 1)* is normally required for the CC-NB-LRR proteins although there are exceptions (Wiermer et al., 2005). *RAR1 (REQUIRED FOR MLA12 RESISTANCE), SGT1 (SUPPRESSOR OF THE G2 ALLELE OF SKP1)* and *HSP90 (HEAT SHOCK PROTEIN 90)* 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 which 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 mis-regulation 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 *acd (accelerated cell death), cpr (constitutive expressor of PR genes), lsd (lesion simulating disease) and ssi (suppressor of SA insensitivity)* (Lorrain et al., 2003) in *Arabidopsis* and *mlo (mutation-induced recessive alleles)* in barley (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., 2006). 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., 2006). 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., 2006). 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., 2006). 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., 2006), 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., 2006). Furthermore, the loss of function of all *BON1* family (*BON1, BON2, BON3*) results in seedling lethality which is largely suppressed by *eds1, pad4* but not by *snc1* or *nahG* (Yang et al., 2006). 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. It however 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 proapoptotic gene Bax and superoxide-generating paraquat in plants. In addition, expressing BAP1 or BAP2 in yeast attenuates cell death induced by hydrogen peroxide (H2O2). 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 thaliana and we named it as BAP2. Using RT (reverse transcription)-PCR (polymerase chain reaction), 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 (Figure 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 TAIR links (http://arabidopsis.org/). BAP2 is under a similar regulation at the transcript level as BAP1. Both genes are upregulated by infections from Botrytis cinerea, nematode, and Pseudomonas syringae, treatments of chemicals (AgNO3, chitin, cycloheximide, ozone, syringolin), and salt stress. They are also both upregulated 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., 2006) and data not shown ).

To assess the spatial expression pattern of BAP2, we fused the promoter of BAP2 with the GUS (ß-glucuronidase) 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 (Figure 1B). This pattern resembles that of pBAP1::GUS (Figure 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 Col-0 (referred as Col from now on), *p35S::BAP2* transgenic lines in *bap1-1* are essentially wild-type in appearance (Figure 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., 2006). 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. Co-expression 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 (Figure 1D), indicating a direct interaction between the BON1 and BAP2 proteins.

Because BON2 and BON3 have overlapping functions with BON1 (Yang et al., 2006), we further determined whether BAP1 and BAP2 can interact with BON2 or BON3 in the yeast two-hybrid system. Co-expression of GBD:BAP1 or GBD:BAP2 with GAD:BON2A and GAD:BON3A respectively conferred yeast growth on the selection medium (Figure 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 (Figure 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 \textit{bap2} from now on), did not exhibit any obvious growth defects, in contrast to the \textit{bap1} mutant (Figure 1E). However, an accelerated HR was observed in \textit{bap2} compared to Col for \textit{Pst} DC3000 expressing AvrRpt2. Col wild type and \textit{bap2} were inoculated with a high concentration of \textit{Pst} DC3000 carrying \textit{avrRpt2}. At 8 hours post inoculation (hpi), none of the Col leaves showed HR, while 50\% of the \textit{bap2} leaves already had HR at this time (Figure 1F and G). At 12 hours 90\% of the \textit{bap2} leaves exhibited HR while only 10\% of the wild type leaves showed HR (Figure 1G).

To reveal possible overlapping functions between \textit{BAP1} and \textit{BAP2}, we attempted to generate double mutants between \textit{bap2} and \textit{bap1}. However, plants with the \textit{bap1bap2} genotype could not be identified from the F2 progenies of a cross between \textit{bap1} and \textit{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 \textit{bap1bap2/+} and 52 out of 194 from \textit{bap1/+bap2} seeds germinated but soon died at the cotyledon stage (Figure 1H). Again, no surviving seedlings were \textit{bap1bap2}, confirming that the double mutant is seedling lethal.

We observed dominant interactions between the \textit{bap1} and \textit{bap2} mutants. \textit{bap1} is a recessive mutant with a mild growth defect (Yang et al., 2006) and \textit{bap2} has no obvious growth defect. However, heterozygous mutants of \textit{bap1} and \textit{bap2} each enhanced the phenotypes of the homozygous mutants of the other (Figure 1I). The \textit{bap1/+bap2} mutant had small and slightly curly leaves in contrast to the wild-type looking \textit{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 \textit{bap1bap2/+} mutant exhibited a stronger phenotype than the \textit{bap1} single mutant. Its leaves are very curly with water soaked appearance, resembling those of \textit{bon1}. The genetic interactions between \textit{BAP1} and \textit{BAP2} indicate that these two genes have overlapping functions and that their functions are dosage dependent.

\textbf{Cell death occurs in mutant combinations between \textit{bap1} and \textit{bap2}}

We assessed cell death in different mutant combinations between \textit{bap1} and \textit{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 (Figure 2A). None of the wild-type
Col leaves (0/8) analyzed had any staining, neither did the \textit{bap1} (0/8) or the \textit{bap2} (0/8) single mutants. Strong staining was found in most of the leaves of \textit{bon1-1} (9/14), consistent with previous findings (Jambunathan et al., 2001; Yang et al., 2006). Very few leaves of \textit{bap1/+bap2} (1/8) were stained by trypan blue, while most of the \textit{bap1bap2/+} leaves (7/12) were stained. Thus, extensive cell death occurs in leaves of \textit{bap1bap2/+} as in \textit{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, \textit{bon1}, \textit{bap1}, \textit{bap2}, or \textit{bap1/+bap2} (Figure 2B). In contrast, strong autofluorescence was found in \textit{bap1bap2/+} (Figure 2B), indicating extensive cell death in \textit{bap1bap2/+}.

We then asked whether the cell death phenotype in \textit{bap1} and \textit{bap2} mutant combinations was associated with an accumulation of ROS. To this end, we determined the relative amount of \( \text{H}_2\text{O}_2 \) in mutant plants by DAB (diaminobenzidine) that forms reddish brown precipitates when reacted with \( \text{H}_2\text{O}_2 \). Under growth conditions of both constant light and 12 hour light/12 hour darkness, \textit{bap1}, but not \textit{bap2}, had a darker staining compared to the wild-type Col. \textit{bap1/+bap2} and \textit{bap1bap2/+} both had a stronger staining than \textit{bap1} (Figure 2C). Thus, \( \text{H}_2\text{O}_2 \) accumulates at a moderate level in \textit{bap1} and at a higher level in the \textit{bap1} and \textit{bap2} mutant combinations.

### Modulation of the \textit{bap1bap2} double mutant phenotypes by \textit{eds1}, \textit{pad4} and the environment

The lethal phenotype of \textit{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 \textit{bap1bap2} is due to a stronger activation of \textit{SNC1} and higher accumulation of SA in the double mutant than in the \textit{bap1} single mutant, given that the loss-of-function mutant \textit{snc1-11} (referred as \textit{snc1} from now on) and the SA degrading \textit{nahG} suppressed the phenotype of \textit{bap1}. Analysis of progenies of a \textit{bap1bap2/+snc1/+} plant and those of a \textit{bap1bap2/+nahG/+} plant indicate that neither \textit{snc1} nor \textit{nahG} could rescue the lethal phenotype of \textit{bap1bap2} (data not shown).

Strikingly, the lethality of \textit{bap1bap2} can be suppressed by mutations in \textit{PAD4} or \textit{EDS1}. From the F2 progenies of a cross between \textit{bap2} and \textit{bap1pad4} (Yang et al., 2006), we were able to obtain \textit{bap1bap2} plants and these plants were always \textit{pad4} homozygous, indicating that \textit{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 (Figure 2D). Similar rescue of lethality of bap1bap2 was observed with the eds1 mutation as well (Figure 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 (Figure 2B). Nor was a higher level of DAB staining observed in bap1bap2pad4, indicating a suppression of H$_2$O$_2$ 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 28ºC 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 28ºC in contrast to the dwarf phenotype at 22ºC (Figure 2E). The bap1bap2 homozygous mutant was partially rescued by a higher growth temperature. Instead of dying immediately after germination at 22ºC, the double mutant grew like the wild type at 28ºC for two weeks after germination. However, when the wild type started bolting at approximately three-week-old, the double mutant turned yellow and died (Figure 2E).

A shorter photoperiod suppressed phenotypes of some of the mutant combinations as well. bap1/+bap2 and bap1bap2/+ grown under a cycle of 12 hr light and 12 hr 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 *Pseudomonas syringae* and *Hyaloperonospora parasitica* (Yang et al., 2006), we assessed whether bap2 has an abnormal defense response. We challenged the bap2 mutant with a virulent bacterial pathogen *P. syringae pv tomato* (Pst) DC3000 and found that it was as susceptible to this pathogen as the wild-type Col (Figure 3A). Four days after infection, Pst grew to 4.2×10$^5$ colony forming unit (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$^7$ in bap1. bap2 was also as susceptible to virulent *H. parasitica* as the wild
type. While no sporangiphores were found on \textit{bap1} a week after spray inoculation, \textit{bap2} supported the same amount of growth of this pathogen as the wild-type Col (Figure 3B).

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

**Overexpression of \textit{BAP} and \textit{BON} genes inhibits PCD induced by a variety of biotic and abiotic stimuli in plants**

The loss-of-function phenotypes indicate that the \textit{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 \textit{Pst DC3000} carrying avirulent effectors in \textit{Arabidopsis}. Wild-type Col plants were infiltrated with \textit{Pst DC3000 (avrRpt2)} together with \textit{Agrobacterium} containing \textit{p35S::BON1}, \textit{p35S::BAP1} or an empty vector. At 14 hpi, a strong HR indicated by the collapse of tissues appeared on all leaves inoculated with \textit{Pst DC3000 (avrRpt2)} together with the vector control (Figure 4A). Agroinfiltrations with \textit{p35S::BAP1} or \textit{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 \textit{p35S::BAP1} and \textit{p35S::BON1} were simultaneously agroinfiltrated (Figure 4A), and it only started to develop at approximately 18 hpi, indicating that \textit{BAP1} and \textit{BON1} together inhibited HR induced by \textit{avrRpt2}.

We additionally tested the effect of \textit{BAP1} and \textit{BON1} overexpression of HR induced by another avirulent strain \textit{Pst DC3000 (avrRPM1)}. At 5-6 hpi, a strong HR was induced by \textit{avrRPM1} when agroinfiltrated with the vector control. Agroinfiltration with \textit{p35S::BAP1} or
p35S::BON1 alone did not significantly affect the development of HR. However, HR was not observed until 8-9 hpi with simultaneous agroinfiltration of BAPI and BON1 (Figure 4A). The suppression for both avirulent strains was consistently seen in replicated experiments. Therefore, overexpression of BON1 and BAPI together in Arabidopsis greatly delayed HR induced by avirulent bacterial pathogen Pst DC3000 with two different effector proteins.

We subsequently analyzed the effect of over-expression of BAPI and BON1 on PCD induced by other R proteins. Transient co-expression of a potato 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., 1999). A collapse of cells indicative of HR was observed in leaf area agroinfiltrated with Rx and CP at 36 hpi. Co-agroinfiltration with the vector alone did not alter the onset or the progression of HR. However when p35S::BAPI or p35S::BON1 were co-agroinfiltrated, HR was either suppressed or greatly reduced at 36 hpi (Figure 4B). In some repeats, no HR was ever developed over the following five days' observation. Co-agroinfiltration of p35S::BAPI and p35S::BON1 together did not appear to have a stronger effect in HR suppression.

Given that BAPI and BON1 inhibit HR induced by R proteins, we further tested whether the BAPI 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 (Figure 4C). Co-agroinfiltration with either p35S::BAPI 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 (Figure 4C). Strikingly, when p35S::BAPI 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 (Figure 4C). Similar suppression of cell death was observed when p35S::BAP2 and p35S::BON1 were co-agroinfiltrated. In both cases, cell collapse started at 90 hpi and occurred to a full extend at 114 hpi in BON1 and BAPI/BAP2 co-infiltrated areas. Therefore,
Bax-induced cell death was delayed by one to two days with over-expression 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 the BAP genes 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., 2006) 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 chlorosis over two days, while leaf discs of 35S::BAP1 transgenic lines stayed green under the same treatment (Figure 4D), indicating a protective role of BAP1 against ROS.

We further asked whether the BAP genes can protect non-plant species from ROS induced cell death. We expressed the BAP1 and BAP2 genes under the control of the constitutive ADH promoter in Saccharomyces cerevisiae, and assayed their effects on cell death induced by ROS. Yeast cells were treated with 10 mM of H2O2 to induce PCD and cell survival rates were counted 12 hours after the treatment. Only 1% of cells containing an empty vector survived the H2O2 treatment compared to the mock treatment (Figure 4E). In contrast, cells expressing either BAP1 or BAP2 had significantly higher survival rates (Figure 4E). 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 H2O2 as well as in similar experiments treated with 5 mM of H2O2 (data not shown).

**Discussion**

**Interaction among the BAP and BON genes**
In this study, we characterized the function of \textit{BAP1} and \textit{BAP2}, two homologous genes encoding small C2 domain-containing proteins. In contrast to the single \textit{bap1} mutants that exhibit a constitutive defense response phenotype, the \textit{bap2} single mutant does not show any obvious growth defects or enhanced disease resistance. \textit{bap2} did exhibit an accelerated HR to an avirulent pseudomonas strain, suggesting that \textit{BAP2} has a role in modulating programmed cell death. Furthermore, the \textit{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 \textit{BAP1} and \textit{BAP2} have unequal redundancy with \textit{BAP1} playing a major role. They also indicate that the amount of activities conferred by \textit{BAP1} and \textit{BAP2} are critical for the process they regulate. This activity decreases roughly in the order of \textit{BAP1BAP2}, \textit{BAP1bap2/+}, \textit{BAP1bap2}, \textit{bap1/+BAP2}, \textit{bap1/+bap2/+}, \textit{bap1/+bap2}, \textit{bap1BAP2}, \textit{bap1bap2/+}, and \textit{bap1bap2}, and it correlates with an increase in morphological phenotypic severity from wild-type to lethality. Considering that expressing \textit{BAP2} under the CaMV 35S promoter rescued the \textit{bap1} single mutant phenotype, we suspect that the \textit{BAP2} might have a lower biological activity than \textit{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 \textit{BAP1} is a functional partner of \textit{BON1}, and it further indicates that the BAP proteins are functional partners of the \textit{BON} proteins. Over-expressing \textit{BAP1} and \textit{BON1} together but not singly inhibits HR induced by avirulent \textit{Pst} and cell death induced by \textit{Bax}, indicating that the \textit{BAP1} and \textit{BON1} proteins work together to modulate cell death. In addition, the loss of the \textit{BAP} family function results in seedling lethality similarly to the loss of the \textit{BON} family function, and the lethality can both be suppressed by \textit{eds1} and \textit{pad4}. Thus the \textit{BAP1} family and the \textit{BON1} family carry similar functions. Nevertheless, the suppression by \textit{eds1} and \textit{pad4} is more complete for \textit{bap1bap2} than for \textit{bon1bon2bon3}, suggesting that the \textit{BON} genes might play a greater role than the \textit{BAP} genes in \textit{Arabidopsis}.

The fact that \textit{BAP1} and \textit{BON1} proteins could interact with each other raises the question whether there are specific pairs of interaction between the \textit{BON} proteins and the \textit{BAP} proteins. The yeast two-hybrid assay demonstrated that each protein of the \textit{BON} family can interact with each member of the \textit{BAP} family although some interactions appear to be stronger than others. This suggests that in plants there could be multiple interactions between the \textit{BON} and \textit{BAP} proteins. This hypothesis is supported by the observation that the \textit{bon1bap1} double mutant has a
stronger phenotype than the bon1 or bap1 single mutants (unpublished results). 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., 2006). 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 programmed cell death across the kingdoms. The loss of function of some of the BAP and BON genes (singly or in combination) leads to micro-lesions, 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., 2006). 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 apoptotic Bax gene, and reactive oxygen species-generating chemical paraquat. More strikingly, cell death in yeast induced by H2O2 is inhibited by BAPI 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 H2O2 in PCD, indicated by their suppression of H2O2 induced cell death in yeast. It is supported by the
observation that overexpression of \textit{BAP1} and \textit{BON1}, though inhibits HR, did not appear to alter the onset of \textit{H}_{2}O_{2} production during \textit{Bax} induced cell death (unpublished results).

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

The dual function of \textit{BAP} and \textit{BON} genes in cell death and defense responses, similarly observed in \textit{MLO} and \textit{LSD} among others, probably reflects an intrinsic relationship between these two processes as exemplified by HR being an integral part of most of the \textit{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 which 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; Little et al., 2006; Yang et al., 2006). 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., 2006), and unpublished results), 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 programmed cell death in other kingdoms.
Materials and Methods

Plant material and growth conditions

*Arabidopsis thaliana* plants were grown at 22°C or 28°C under continuous fluorescent light (100 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) with 50-70% 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 hours of light for two weeks (for dipping inoculation) or one 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 vector pGBD-C2 with a tryptophan 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 leucine auxotroph maker (James *et al.*, 1996). pGBD:BAP1 and pGBD:BAP2 were each co-transformed 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 tryptophan and leucine. Protein interactions were assayed by growing the transformants on SC medium without adenine, histidine, tryptophan, and leucine.

Northern blot analysis

Total RNAs were extracted from 3-week-old plants using TriReagents (Molecular Research Inc., Cincinnati, OH) 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 KB 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 one hour. The amount of bacteria in plants was analyzed at one hour after dipping (day 0) and 4 days 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 cfus 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 thaliana*. 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 MgCl2, 10 mM MES, and 150 µM Acetosyringone) at 0.5 OD600. Cells with Rx or CP were resuspended at 0.2 OD600 and combined at 1:1 to make the Rx and CP mixture. Cells containing the pDEX:Bax were resuspended at 0.5 OD600 2 hours prior to infiltration. 50 µM of DEX was sprayed onto N. benthamiana leaves 15 hours 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 50mM of Tris-acetate (pH5.0) at a concentration of 1mg/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 hours before the tissues were cleared in boiling ethanol (95%) for 10 minutes.

For paraquat treatment, leave disks from 3-week-old plants were floated on 4 µM of paraquat. They were first kept in dark for 1 hour and then incubated under light for two to three days.

**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 S. cerevisiae 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-leucine) for 36 hours, collected by centrifugation, washed 3 times with water, and resuspended in fresh medium at a concentration of 0.5 OD600. Each
sample was split into two halves with one treated with H$_2$O$_2$ 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 hours after treatment was analyzed by growing serial dilutions onto rich media.

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Figure Legends

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 synthetic complete (SC) medium selecting for protein-protein interaction three days 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 three-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 hours post inoculation (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 hours after inoculation (G). Replicated experiment 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.
Figure 2. Cell death occurs in \textit{bap1} and \textit{bap2} mutant combinations. 
(A) Trypan blue staining of representative leaves of Col, \textit{bon1}, and \textit{bap1bap2/+}. In contrast to wild-type Col and the \textit{bap1} and \textit{bap2} single mutants (data not shown), \textit{bap1bap2/+} has a strong trypan blue staining similar to \textit{bon1}.
(B) Autofluorescence of leaves from Col, \textit{bap1}, \textit{bap2}, \textit{bon1}, \textit{bap1bap2/+}, \textit{bap1/+bap2}, \textit{bap1bap2pad4} and \textit{bap1bap2eds1}. \textit{bap1bap2/+} has the strongest autofluorescence while the \textit{bap1} and \textit{bap2} single mutants have no significant amount. Autofluorescence is absent in \textit{bap1bap2 pad4} and \textit{bap1bap2eds1}.
(C) Accumulation of H$_2$O$_2$ in various mutants. Upper panel shows DAB staining of two-week-old plants grown under constant lights and the lower panel shows DAB staining of individual leaves from plants grown under 12 hours light and 12 hours of darkness. Note the weak staining in \textit{bap1}, a strong staining in \textit{bap1bap2/+}, \textit{bap1/+bap2}, but no staining in wild-type Col, \textit{bap2}, \textit{bap1pad4}, and \textit{bap1bap2pad4}.
(D) Both \textit{pad4} and \textit{eds1} rescued the lethal phenotype of \textit{bap1bap2}. Shown are 3-week-old seedlings of the wild-type Col, \textit{bap1bap2pad4} and \textit{bap1bap2eds1} grown at 22ºC. \textit{bap1bap2} was dead at this stage.
(E) High temperature partially rescued the \textit{bap1bap2} mutant phenotype. Shown are 3-week-old seedlings of the wild-type Col, \textit{bap1/+bap2}, \textit{bap1bap2/+} and \textit{bap1bap} grown at 28ºC. Note \textit{bap1bap2/+} and \textit{bap1/+bap2} are wild-type looking and \textit{bap1bap2} is surviving but yellowing at this stage.

Figure 3. The \textit{bap2} mutant does not have an enhanced disease resistance.
(A) \textit{bap2} is susceptible to \textit{Pseudomonas syringe pv tomato} DC3000. Plants were infected with \textit{Pst} DC3000 and the amount of bacterial growth in the leaves was determined at 0 and 4 days post inoculation (dpi). Bacterial growth was inhibited in \textit{bap1} but not in \textit{bap2} compared to the wild-type Col. \textit{bap1/+bap2} and \textit{bap1 bap2/+} had approximately the same amount of growth as \textit{bap2} and \textit{bap1} respectively. \textit{bap1bap2pad4} supports the same amount of bacterial growth as the \textit{pad4} single mutant.
(B) \textit{bap2} is susceptible to virulent \textit{Hyaloperononspora parasitica}. \textit{H. parasitica} Noco2 strain was used to infect Col, \textit{bap1}, and \textit{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.

Figure 4. Overexpression of *BAP1* and *BAP2* suppresses programmed cell death (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 hypersensitive response (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 co-agroinfiltrated 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 dexamethasone. 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 co-expression of *BAP1* or *BAP2* together with *BON1*.
(D) *BAP1* overexpression confers paraquat resistance. Leaf disks from the wild type Col and *35S::BAP1* transgenic plants were floated on paraquat solution (4 µM) for two days before pictures were taken.
(E) Yeast strains transformed with *BAP1*, *BAP2* or the empty vector pAD4M were treated with 10 mM of H₂O₂ or H₂O (mock). Shown is the amount of live cells 12 hours 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$_2$O$_2$. 
A

BAP1 1
BAP2 1

BAP1 1
BAP2 1

BAP1 51
BAP2 41

BAP1 98
BAP2 91

BAP1 145
BAP2 141

BAP1 176
BAP2 191

B

pBAP1::
GUS

pBAP2::
GUS

C

Col  bap1-1  p35S::BAP2  bap1-1

D

GAD
GAD: BON1A
GAD: BON2A
GAD: BON3A

GBD:
BAP1
GBD:
BAP2

E

Col  bap1-1  bap2-4

F

Col  bap2-4

G

leaves showing HR (%)

Hours post inoculation

H

bap1  bap1  bap2

I

Col  bap1  bap2  bap2/+
