BON1 interacts with the protein kinases BIR1 and BAK1 in modulation of temperature-dependent plant growth and cell death in Arabidopsis

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SUMMARY

The Arabidopsis copine gene BON1 encodes a calcium-dependent phospholipid-binding protein involved in plant growth homeostasis and disease resistance. However, the biochemical and molecular mechanisms by which BON1 modulates plant growth and defense responses are not well understood. Here, we show that BON1 interacts physically with the leucine-rich-repeat receptor-like kinases BIR1 (BAK1-interacting receptor-like kinase 1) and pathogen-associated molecular pattern (PAMP) receptor regulator BAK1 in vitro and in vivo. Additionally, bon1 and bir1 mutants exhibit synergistic interaction. While a bir1 null mutant has similar growth and cell-death defects compared with bon1, a bir1 bon1 double mutant displays more severe phenotypes than does the single mutants. The bon1-1 and bir1-1 phenotypes are partially suppressed by overexpression of BIR1 and BON1, respectively. Furthermore, the bir1 phenotype is attenuated by a loss-of-function mutation in the resistance (R) gene SNC1 (Suppressor of npr1-1, constitutive 1), which mediates defense responses in bon1. Intriguingly, BON1 and BIR1 can be phosphorylated by BAK1 in vitro. Our findings suggest that BIR1 functions as a negative regulator of plant resistance and that BON1 and BIR1 might modulate both PAMP- and R protein-triggered immune responses.

Keywords: BON1, receptor-like kinases BIR1 and BAK1, growth, cell death, Arabidopsis.

INTRODUCTION

The copines are a family of highly evolutionarily conserved proteins that are found in protozoa, nematodes, mammals, and plants (Creutz et al., 1998). Copine proteins are characterized by two C2 domains at the N-terminus and a von Willebrand A domain at the C terminus. The C2 domains are calcium-dependent phospholipid-binding domains found mostly in signal transduction and membrane trafficking molecules (Rizo and Sudhof, 1998). The A domain of human copines associates with various proteins, such as mitogen-activated protein kinase kinase 1, protein phosphatase 5, and the NEDD8-conjugating enzyme UBC12 (Tomsig et al., 2003, 2004). The A domain of human copine III has also been shown to possess intrinsic kinase activity (Caudell et al., 2000). Copine structures and activities suggest that they may play crucial roles in membrane trafficking and signal transduction.

Genetic studies have revealed that the Arabidopsis copine gene BON1 affects temperature-dependent plant growth homeostasis and defense responses (Hua et al., 2001; Jambunathan et al., 2001). BON1 is a repressor of the disease resistance (R) gene SNC1 (Suppressor of npr1-1, constitutive 1) (Yang and Hua, 2004). SNC1 encodes a TIR-NB-LRR protein, which is a member of the RPP4/RPP5 protein family (Zhang et al., 2003). The loss of BON1 function results in activation of SNC1 and subsequent temperature-dependent growth defects (Yang and Hua, 2004). BON1 has two homologs in Arabidopsis, BON2 and BON3, that have redundant functions essential for plant viability (Yang et al., 2008b). The double mutants of bon1 bon2 and bon1 bon3 exhibit extensive cell death and show seedling lethality, both of which can be suppressed either by pad4 and eds1 or by a higher temperature of 28°C (Yang
et al., 2006b). BON1 and BON3 have further been shown to repress a number of R-like genes. At least four R-like genes, including lesion cell death 2 (LCD2), LCD4, LCD6 and LCD7, contribute quantitatively to the cell-death phenotype of the bon1 bon3 double mutant (Li et al., 2009). Therefore, BON genes act as negative components of multiple R gene-triggered pathways to modulate plant growth, defense responses and cell death. Many NB-LRR type R proteins detect pathogen effectors to trigger defense responses, referred to as effector-triggered immunity (ETI) (Chisholm et al., 2006). ETI is often associated with a hypersensitive response (HR), a form of programmed cell death at the infection sites. Different mechanisms have been proposed to explain how the intracellular recognition of effector proteins occurs. The guard hypothesis proposes that an R protein guards the effector-targeted host protein and modification of this target by the effector results in the activation of the R protein (Van der Biezen and Jones, 1998). In the absence of the pathogen effectors, R proteins are kept in an ‘off’ state by negative regulators.

Plants possess an more ancient form evolutionarily of immunity that is triggered by perception of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) at the cell surface (called PAMP-triggered immunity or PTI). Flagellin-sensitive 2 (FLS2) receptor and the elongation factor Tu (EFR) receptor are two well characterized PRRs of PAMPs that belong to the receptor-like kinases (RLK) family (Gomez-Gomez and Boller, 2000; Zipfel et al., 2006). Plants contain large numbers of RLKs composed of an extracellular ligand-binding domain, a transmembrane domain, and a cytoplasmic serine/threonine kinase domain. The extracellular domains of these RLKs are quite divergent, and allow different RLKs to respond selectively to different signals. Receptor-like kinases that contain leucine-rich-repeats (LRR-RLKs) are the largest group of RLKs, with at least 223 members in the Arabidopsis genome (Shiu et al., 2004). Several LRR-RLKs are implicated in modulation of plant growth and development (Li and Chory, 1997; Jinn et al., 2000) and plant defense responses (Gomez-Gomez and Boller, 2000; Shiu et al., 2004; Morillo and Tax, 2006; Zipfel et al., 2006; Afzel et al., 2008). For example, two LRR-RLKs, brassinosteroid insensitive 1 (BRI1) and BRI1-associated receptor kinase 1 (BAK1), are co-receptors for the plant hormone brassinosteroid (BR) and control positively the early events of BR signaling (Li and Chory, 1997; Li et al., 2002; Nam and Li, 2002). FLS2 and EFR recognize the peptides from bacterial flagellin (flg22) and from bacterial translation elongation factor EF-Tu, respectively. Upon flg22 perception, BAK1 and FLS2 physically associate and then both interact with the receptor-like cytoplasmic kinase BIK1 (Botrytis-induced kinase 1), thus initiating plant innate immunity (Chinchilla et al., 2007; Lu et al., 2009). BAK1 also functions as a negative regulator in a BR-independent cell-death pathway (He et al., 2007; Kemmerling et al., 2007). Two additional LRR-RLKs, BIR1 (BAK1-interacting receptor-like kinase 1) and SOBIR1 (Suppressor of bir1-1), were recently identified as regulators of multiple resistance signaling pathways (Gao et al., 2009). The loss of function of BIR1 induces cell death and constitutive defense responses that are suppressed by a mutation in SOBIR1. Overexpression of SOBIR1 leads to cell death and activates defense responses (Gao et al., 2009).

There are likely to be interconnections between PTI and ETI, but the molecular bases of these are not well characterized. Here, we report identification of the LRR-RLK BIR1 and BAK1 as BON1-interacting proteins both in vitro and in vivo. Genetic analyses revealed that bir1 phenotypes were suppressed partially by a loss-of-function snc1 mutation. Additionally, bir1 and bon1 mutant phenotypes were suppressed partially by overexpression of BON1 and BIR1, respectively. Furthermore, BON1 and BIR1 can be phosphorylated by BAK1 in vitro. Thus, BON1 might be involved in BIR1 and BAK1 signaling, which suggests a connection between RLK-mediated PTI and R protein-mediated ETI modulated by BON1.

RESULTS

Identification of an LRR-receptor kinase as a putative binding partner of BON1

Previous studies have revealed that the copine gene BON1 regulates temperature-dependent plant growth homeostasis and defense responses (Hua et al., 2001; Jambunathan et al., 2001; Yang and Hua, 2004). To further investigate the biological process in which BON1 is involved, we screened for proteins that interact with BON1 using a yeast two-hybrid assay. The C-terminus A domain (BON1-A, from Val 319 to Pro 578), which is involved potentially in protein–protein interactions (Creutz et al., 1998; Hua et al., 2001), was fused to the yeast GAL4 binding domain and then used as bait to screen a cDNA library that had been constructed from the vegetative tissues of 3-week-old Arabidopsis plants obtained from the Arabidopsis Biological Resource Center (ABRC). Approximately $1.0 \times 10^6$ yeast transformants were screened on a synthetic complete (SC) medium that lacked leucine, tryptophan, adenine, and histidine. Two of more than 20 positive cDNA clones identified correspond to 552 bp of the 3’ portion of At5g48380 that encodes the BIR1 protein (Gao et al., 2009).

BIR1 is a typical RLK with a presumed extracellular domain, a single transmembrane domain, and a cytoplasmic serine/threonine kinase domain (Figure S1). The predicted extracellular domain contains discrete regions that include a cleavable N-terminal signal peptide and two cysteine pairs flanking four LRRs (Figure S1). The intracellular domain is composed of an intracellular juxtamembrane (JM) domain, a catalytic kinase domain, and a C-terminal domain. BIR1 belongs to the LRR-X RLK subfamily in the Arabidopsis
genome classification (Shiu and Bleecker, 2001) and shares sequence similarity with three other putative RLKs (At1g27190, At1g69990, and At3g28450) (Figure S1).

The kinase domain of BIR1 is necessary for interaction between BIR1 and BON1

To determine which domain of BIR1 interacts with BON1, we generated a series of cDNA constructs in which the full-length, or truncated coding sequences of BIR1 were fused translationally with the activation domain of the GAL4 transcription factor known as GAD. These constructs include full-length BIR1 (BIR1-FL), the entire intracellular domain of BIR1 (BIR1-JKC), the intracellular domain with both the C-terminal and JM domains deleted (BIR1-KD), and the intracellular C-terminal domain (BIR1-CT) (Figure 1a). The resulting constructs were transformed into yeast cells that expressed the BON1-A domain fused with the DNA-binding domain of GAL4 (GBD). BIR1-JKC and BIR1-KD were found to interact with BON1-A in the yeast two-hybrid assay. In contrast, BIR1-CT did not exhibit an interaction with BON1 (Figure 1b). BIR1-FL was unable to interact with BON1-A in our yeast two-hybrid assay, probably because BIR1 is a transmembrane protein that cannot be expressed effectively in the nucleus. These results demonstrate that the kinase domain of BIR1 is necessary for the interaction between BIR1 and BON1.

BIR1 interacts with BON3 proteins in yeast

BON1 has two closely related homologs, BON2 and BON3, in Arabidopsis (Yang et al., 2006b). We tested whether BIR1 might also interact with BON2 and BON3 using the yeast two-hybrid system. Co-expression of the A domain of BON3 and BIR1-JKC conferred yeast host strain growth on the SC/-Leu/-Trp/-Ade/-His selection medium as well as β-galactosidase activity (Figure 1c). However, the yeast strain that co-expressed the A domain of BON2 and BIR1-JKC could not grow on the SC/-Leu/-Trp/-Ade/-His selection medium. This finding indicates that BIR1 interacts with BON3, but not BON2, in yeast. It is also possible that the interaction between BIR1 and BON2 in yeast is too weak for detection.

BIR1 interacts with BON1 in vitro and in vivo

Previous studies have shown that BON1 and BIR1 are plasma-membrane-associated proteins (Hua et al., 2001; Gao et al., 2009), which suggests that the subcellular localization of BON1 and BIR1 overlaps in plants.

The interaction between BIR1 and BON1 was verified by in vitro binding assays. The BON1-A domain fused to maltose binding protein (MBP) and the BIR1-JKC domain fused to glutathione S-transferase (GST) were expressed in *E. coli* and used in an in vitro pull-down assay. The recombinant BIR1-GST protein was able to pull down the BON1-MBP protein, but not MBP alone (Figure 2a).

Additionally, GST did not pull down the BON1-MBP protein (Figure 2a). These data indicate that BIR1 and BON1 proteins interact physically *in vitro*.

We next tested the BIR1 and BON1 protein interactions in plant cells using a bimolecular fluorescence complementation (BIFC) assay in Arabidopsis protoplasts and *Nicotiana benthamiana* leaves (Walter et al., 2004; Yoo et al., 2007). BON1 was fused to an N-terminal yellow fluorescent protein fragment (YFP<sup>N</sup>), and BIR1 was fused to a C-terminal YFP fragment (YFP<sup>C</sup>). When BON1-YFP<sup>N</sup> was...
coexpressed with BIR1-YFP\(^{C}\) in Arabidopsis protoplasts or \(N.\) benthamiana leaves, a bright fluorescence signal was observed on the plasma membrane (Figures 2b, S2). In contrast, leaves that were co-transformed with BON1-YFP\(^{N}\) and the RLK CLV1-YFP\(^{C}\) failed to yield any YFP signal (Figure 2b). Neither the co-expression of BON1-YFP\(^{N}\) and unfused YFP\(^{C}\) nor the co-expression of BIR1-YFP\(^{C}\) and unfused YFP\(^{N}\) yielded any detectable fluorescence (Figure S2). Together, these results indicate that BIR1 and BON1 interact with each other at the plasma membrane.

We also performed a co-immunoprecipitation (Co-IP) experiment using BON1-HA and BIR1-myc expressed transiently in Arabidopsis protoplasts. The BIR1-myc protein was immunoprecipitated using anti-myc conjugated agarose. Immunoblots were washed and then probed with anti-HA antibodies. The BON1-HA protein was pulled down by BIR1-myc (Figure 2c). In contrast, immunoprecipitation from protein extracts of Arabidopsis protoplasts that expressed BON1-HA produced no cross-reactivity with anti-HA (Figure 2c). These findings indicate, therefore, that BIR1 and BON1 can occur in the same complex.

### Expression pattern of \(BIR1\)

To determine the tissue-specific expression of \(BIR1\), a 1.2 kb genomic fragment upstream of the \(BIR1\) translational start codon was amplified and cloned into the binary vector \(p\)ZPGUS2 that was fused transcriptionally to the \(\beta\)-glucuronidase (\(GUS\)) reporter gene. Of the 23 independent transgenic lines generated, 18 lines showed similar \(GUS\) expression patterns. One representative line was selected to analyze \(BIR1\) expression patterns further. \(BIR1::GUS\) signals were detected in leaves, roots, and flowers (Figure 3a). The ubiquitous expression was confirmed by quantitative real-time polymerase chain reaction (PCR). \(BIR1\) was found to be expressed in almost all tissues tested, with the highest expression in leaves and the lowest in stems (Figure 3b). Furthermore, \(BIR1\) expression was not affected obviously by the pathogen \(Pseudomonas syringae\) pv. \(tomo(\,\,\) (\(Pst\) DC3000, which can induce \(BON1\) expression (Figure 3c).

\(bir1\) mutants show \(bon1\) \(bon2\)- and \(bon1\) \(bon3\)-like morphological phenotypes

We obtained two \(BIR1\) T-DNA insertion lines designated as \(bir1\)-1 and \(bir1\)-2 to examine the genetic interaction between \(BIR1\) and \(BON1\) further (Figure 4a). RT-PCR analyses were performed to determine whether the expression of \(BIR1\) was abolished in these two lines. The amounts of full-length \(BIR1\) transcripts were greatly reduced in both alleles compared with the wild type, and the expression in the \(bir1\)-2 was slightly higher than that found in \(bir1\)-1 (Figure 4b-c). Because these two alleles showed similar growth defects (Figures 4d and S3a), we chose \(bir1\)-1 for further analyses; this allele is the same as reported by Gao et al. (2009). The \(bir1\) mutants showed no obvious defects compared with wild-type plants up to 4 days after germination. However, the \(bir1\) mutants exhibited delayed and reduced leaf expansion compared with wild-type plants and died before bolting. Lateral root growth in \(bir1\) mutants was also...
severely inhibited (Figure 4d). We found that the BIR1 cDNA fused with green fluorescent protein (GFP) driven by the CaMV 35S promoter (35S::BIR1-GFP) rescued the growth defect phenotype of bir1-1 (Figure 4e), which was consistent with a previous study showing that a genomic BIR1 fragment with its native promoter complemented the bir1-1 phenotype (Gao et al., 2009).

The growth arrest and lethality observed for bir1-1 at 22°C were similar to observations on the bon1 bon2 and bon1 bon3 double mutants (Yang et al., 2006b) (Figures 4e and S3b). Additionally, similar to the bon1 bon3 double mutant (Yang et al., 2006b), the phenotypes of bir1-1 were rescued partially by the high temperature of 28°C (Figure 4e). Although the bir1-1 plants were smaller than Col or bon1-1, they had greatly expanded leaves and could bolt and set seeds occasionally, in agreement with previous observations (Gao et al., 2009).

We generated a bon1 bir1 double mutant for further analysis. The phenotype of the bir1 bon1 double mutant was more severe than that of bon1-1 or bir1-1 single mutants. The bir1 bon1 double mutants exhibited severe leaf curling and reduced growth compared with the bon1-1 or bir1-1 single mutants, and seeding lethality at young stages (Figure 4f, g).

**bir1-1 exhibits bon1 bon2- and bon1 bon3-like cell death and defense phenotypes**

The bir1 plants showed a cell-death phenotype at 22°C (Gao et al., 2009), which resembled what is seen in bon1 bon2 and bon1 bon3 double mutants (Yang et al., 2006b). Trypan blue staining revealed that, as in bon1 bon2 and bon1 bon3 plants, extensive cell death of bir1 mutants was suppressed by the higher temperature of 28°C (Figure 5a). Similar to bon1 bon2 and bon1 bon3 double mutants, bir1 plants displayed enhanced expression of the PR1 and PR2 genes, which was abolished at 28°C (Figure 5b, c).

We further examined the expression of BIR1 and BON1 in bir1-1, bon1-1 and bon double mutants at 22 and 28°C. BON1 was dramatically induced in bir1-1 plants at 22°C, and this induction was compromised partially at 28°C (Figure 5d). BIR1 expression was upregulated significantly in the bon1-1 and the bon double mutants at 22°C, but was nearly abolished at 28°C (Figure 5e). These data suggest that BON1 and BIR1 regulate one another’s expression in a temperature-dependent manner.

**SNC1 contributes to the bir1 phenotype**

The lethality observed in bir1-1 plants results from an activated defense response that leads to extensive cell death at an early stage of plant development. We assessed whether the lethal phenotype of bir1-1 was due to stronger activation of SNC1. As found for bon1-1, SNC1 expression in bir1-1 plants was highly upregulated at 22°C but not at 28°C (Figure 5f).

Next, we generated bir1 snc1-11 double mutants by crossing heterozygous bir1/+ plants with an snc1-11 loss-of-function mutant (Yang and Hua, 2004) and analyzed the progenies of bir1/+ snc1-11 plants. The bir1-1 snc1-11 plants were intermediate in size, and their leaves were not as curled as those of bir1-1 plants (Figure 6a). Furthermore, the extensive cell-death phenotype seen in bir1-1 was suppressed partially by snc1-11 (Figure 6b). Also, the bir1-1 snc1-11 plants exhibited less H₂O₂ accumulation than did bir1-1 plants as revealed by staining with 3′,3-diaminobenzidine.
(DAB) (Figure 6b). Up-regulation of the PR1 and PR2 genes in bir1-1 plants was partially blocked by snc1-11 (Figure 6c), consistent with the morphological and cell-death phenotypes. Therefore, the bir1-conferred phenotypes are dependent partially on SNC1.

**Reciprocal regulation of BON1 and BIR1**

We crossed heterozygous bir1-1/+ plants with 35S::BON1 transgenic plants harboring a BON1 genomic fragment under control of the CaMV 35S promoter (Yang et al., 2007) to further investigate the interaction between BON1 and BIR1. RT-PCR analysis indicated that BON1 was overexpressed in these bir1/35S::BON1 transgenic lines (Figure 7a). The transgenic plants were larger than the bir1-1 plants in terms of morphology, and their leaves were also more extended compared with the bir1-1 plants (Figure 7b). Moreover, both fewer dead cells and less H2O2 accumulation were detected in the leaves of the bir1/35S::BON1 plants compared with the bir1-1 plants (Figure 7c). Furthermore, expression of the PR1 and PR2 genes in bir1-1 was partially suppressed by the overexpression of BON1 (Figure 7d).

We next generated bon1/Super::BIR1 plants by transforming pSuper::BIR1 into the bon1-1 mutant. Eight of 11 independent transgenic lines that overexpressed the BIR1 gene (Figure 8a) exhibited apparent wild-type morphology before bolting (Figure 8b). Accordingly, cell death and H2O2 accumulation were largely abolished in these plants (Figure 8c). The expression of PR2 gene was dramatically reduced in bon1/35S::BIR1 (Figure 8d). After bolting, these 35S::BIR1 bon1 plants displayed a slightly dwarfed stature (Figure 8b). Therefore, the bon1-1 phenotype is suppressed partially by overexpression of BIR1. These results, together with the phenotype of the bon1 bir1 double mutant, suggest reciprocal regulation of BIR1 and BON1.
BON1 interacts with BAK1 in yeast and plants

Because BIR1 interacts physically with the BAK1 protein (Gao et al., 2009), we determined whether BON1 could also associate with BAK1. The intracellular kinase domain of BAK1 (BAK1-KD) was fused with the GAL4 binding domain, and the BON1A domain was fused with the GAL4 activation domain. Yeast two-hybrid assays indicated that BON1 was able to interact with BAK1 (Figure 9a). To examine the in vivo interaction between BON1 and BAK1, we performed a BiFC experiment in Arabidopsis protoplasts. BON1 was fused to YFP\(N\) and BAK1 was fused to YFP\(C\). When BON1-YFP\(N\) was coexpressed with BAK1-YFP\(C\) in Arabidopsis protoplasts, a YFP signal was observed in the plasma membrane (Figure 9b), indicating a close interaction between BON1 and BAK1 in plants. Co-IP analysis of protein extracts from Arabidopsis protoplasts co-transformed with BON1-HA and BAK1-myc also revealed a positive interaction between BON1 and BAK1 (Figure 9c). Taken together, these results indicate that there is direct association among BON1, BIR1 and BAK1.

BAK1 phosphorylates BON1 and BIR1 in vitro

BAK1 was shown to exhibit autophosphorylation in vitro (Gao et al., 2009). We found that the BIR1 cytoplasmic domain fused with glutathione \(S\)-transferase (BIR1-GST) showed autophosphorylation activity in the presence of Mn\(^{2+}\) but not with Mg\(^{2+}\) or Ca\(^{2+}\). The autophosphorylation activity in Mn\(^{2+}\) buffer was not affected significantly by Mg\(^{2+}\) or Ca\(^{2+}\) (Figure 10a). Therefore, recombinant BIR1 is an active Mn\(^{2+}\)-dependent protein kinase.

Because BIR1 and BAK1 are protein kinases that interact with BON1, we asked whether BAK1 and BIR1 phosphorylate BON1. In addition to BIR1-GST, the GST tagged BON1-A
domain (BON1-GST) and the MBP tagged BAK1 cytoplasmic domain (MBP-BAK1KD) and its kinase dead form (MBP-mBAK1, Li et al., 2002) were used in in vitro kinase assays. It was previously reported that the A domain of human copine III exhibits kinase activity (Caudell et al., 2000). Nevertheless, no autophosphorylation activity of the recombinant BON1A protein was detected under the conditions used in our experiments. Moreover, the BON1 protein was not phosphorylated when it was co-incubated with BIR1 (Figure 10b). When BON1 was incubated with BAK1, BON1 was phosphorylated (Figure 10c). In contrast, incubation with kinase dead BAK1 (mBAK1) completely abolished the phosphorylation of BON1 (Figure 10c). As BIR1 was reported to exhibit direct interaction with BAK1 (Gao et al., 2009), we examined whether BIR1 could be phosphorylated by BAK1.

The autophosphorylation activity of K331E mutated (mBIR1) form of BIR1 was shown to be dramatically reduced (Gao et al., 2009) (Figure 10b). Therefore mBIR1 was used in the kinase assay. BAK1, but not mBAK1, was able to efficiently phosphorylate mBIR1 (Figure 10d). Taken together, our data suggest that both BON1 and BIR1 are substrates of BAK1 in vitro.

**DISCUSSION**

The Arabidopsis copine gene BON1 is a negative regulator of plant defense responses, and this regulation occurs largely through R genes (Jambunathan et al., 2001; Yang and Hua, 2004; Li et al., 2009). BAK1 is a coreceptor of a number of PAMPs required for the perception of bacterial flagellin and PAMP signaling (Chinchilla et al., 2007; Heese et al., 2007; Shan et al., 2008). The LRR-receptor protein kinase BIR1 protein is a BAK1-interacting protein that is involved in cell death and innate immunity (Gao et al., 2009). The present study shows that BON1 associates with BIR1 and BAK1 in vivo, and that BIR1, like BON1, impacts SNC1 resistance negatively, which suggests a connection between PTI and ETI.

BON1 and BON3 have been shown to be negative regulators of several TIR-NB-LRR and TIR-NB-type R-like genes, including SNC1, LCD2, LCD4, LCD5, and LCD7 (Yang et al., 2006a; Li et al., 2009). Loss-of-function of SNC1 and these LCD genes compromise quantitatively the cell death and defense phenotypes of the bon1 and bon1 bon3 mutants (Yang et al., 2006b; Li et al., 2009). Thus, BON proteins can be thought of as being guarded by multiple R genes. Because of complete suppression of bir1-conferred phenotypes by pad4-1 and sobir1-1, BIR1 or the protein complex of BIR1 and BAK1 were proposed to be guarded by two or multiple R proteins, one of which belongs to the TIR-NB-LRR class (Gao et al., 2009). In this study, we found that the loss-of-function mutant snc1-11 rescued partially the defense and cell-death phenotypes of the bir1 mutant. Therefore, the phenotype of bir1-1 may be due to inappropriate activation of multiple R-like genes, and BIR1 and BAK1 may be guarded by R-like proteins, such as SNC1 and/or LCDs.

Copines are ubiquitous Ca²⁺-dependent phospholipid-binding proteins that are conserved in animals and plants. Human copine III was identified as a binding partner of ErbB2, which is a member of the type I receptor tyrosine kinase (RTK) family located at the plasma membrane (Heinrich et al., 2009). ErbB2 functions in ligand-activated signaling pathways that regulate cell proliferation and cell death (Citri and Yarden, 2006); copine III has been shown to play important roles in regulating ErbB2-dependent cancer cell motility (Heinrich et al., 2009). RLKs in plants are similar to RTKs in animals in terms of basic structure. In this study, we provided evidence that the plant copine protein BON1 interacts with two RLKs, BIR1 and BAK1, to control R-mediated plant survival and defense responses.
The loss-of-function mutant \textit{bir1} shows phenotypes that resemble \textit{bon1 bon2} and \textit{bon1 bon3} mutants in the following respects. First, extensive cell death occurs, leading to seedling lethality at 22°C. Second, the growth defect is rescued at the higher temperature of 28°C. Third, the lethal phenotype is suppressed by mutations in \textit{PAD4} and \textit{EDS1} to varying extents (Yang \textit{et al.}, 2006b; Gao \textit{et al.}, 2009). A previous study demonstrated that \textit{bak1 bkk1} showed similar cell death and seedling-lethality phenotypes (He \textit{et al.}, 2007). Additionally, these three proteins exhibit physical interactions among each other in plants. Thus, the interaction between copines and receptor kinases at the plasma membrane may be a conserved regulatory mechanism across kingdoms. It is noteworthy that overexpression of \textit{BON1} and \textit{BIR1} results in partial mutual suppression of the loss-of-function phenotypes of the other
gene, and the bon1 bir1 double mutant exhibits more severe phenotypes than each of the single mutants. This finding prompts us to propose that BON1 may interact with BIR1 homologs, and BIR1 may interact with BON2 and BON3. Additionally, the association of BON1 with BIR1 and BAK1, two cell death regulators, is in line with the hypothesis that ectopic expression of the A domain of BON1 exerts a dominant negative effect, probably by interfering the binding of the native BON1 to its binding partners, resulting in cell death (Li et al., 2005).

Human copine III protein has been suggested to be a phosphoprotein that possesses kinase activity (Caudell et al., 2000). Alternatively, this kinase activity might result from a co-purified protein kinase rather than from copine III itself because copine III includes no known kinase motif (Heinrich et al., 2009). In agreement with this latter scenario, we failed to detect kinase activity in BON1 in vitro. Instead, we demonstrated that BON1, as well as BIR1, can be phosphorylated by BAK1 in vitro. As the K331E mutated BIR1 form did not fully abolish its autophosphorylation activity, we cannot exclude one possibility that BAK1 may enhance autophosphorylation of BIR1. It remains to be determined whether the phosphorylation of BON1 is required for the negative regulation of R-gene-mediated resistance pathways. It is possible that mutual regulation exists among BON1, BIR1 and BAK1. BON1 activity could be modulated by BAK1/BIR1, and BON1 may also modulate BIR1 and BAK1 activity.

BON1 is a plasma-membrane localized protein, and it binds to phospholipids in a calcium-dependent manner in vitro (Hua et al., 2001). Its expression is regulated by temperature, SA, and calcium (Hua et al., 2001; Lee and McNellis, 2009) (http://www.arabidopsis.org). BON1 is involved in Ca^{2+} signaling, as has been demonstrated by inducing a defense phenotype with calcium ionophore under permissive conditions (Lee and McNellis, 2009). Structure–function analysis of the BON1 protein revealed that calcium binding is essential for its activity, but not its plasma-membrane localization (Li et al., 2010). Instead, myristoylation of the protein is essential for targeting it to the plasma membrane (Li et al., 2010). Given these findings, together with the data obtained in the present study, BON1 is likely to be an important signal molecule that integrates RLKs and calcium in the signal transduction pathways involved in defense responses and cell death.

In summary, our results suggest a working model in which BON1 and two RLKs, BIR1 and BAK1, mutually modulate one another’s activity to regulate multiple R genes negatively, thus controlling plant survival and cell death in Arabidopsis. BAK1 associates with and may phosphorylate BON1 and BIR1, both of which are negative components of cell death and defense governed by R-like proteins. As BAK1 modulates multiple PAMP receptors, the repression of R genes by BIR1 and BON1 suggests a close connection between ETI and PTI. It is possible that PAMP-triggered immunity by BAK1-associated PAMP receptors may be in part realized by de-repressing R-dependent defense response normally curbed by BON1 and BIR1. Further studies on the biochemical properties of BON1 and BIR1 and their regulation by BAK1 under various environmental conditions should elucidate the intricacies of their regulation of defense responses.

**EXPERIMENTAL PROCEDURES**

**Plant materials and growth conditions**

Col-0 and No-0 accessions of wild-type *Arabidopsis thaliana* were used in this study. Plants were grown at 22 or 28°C under a 16-h light/8-h dark photoperiod at 100 μmol m^{-2} s^{-1} in soil or on Murashige and Skoog (MS) medium (Sigma-Aldrich, http://www.sigmaaldrich.com/) containing 2% sucrose and 0.8% agar. *N. benthamiana* plants were cultivated in a greenhouse at 25°C under a 16-h light/8-h dark photoperiod with 50–60% relative humidity.

The bir1-1 (WiscDsLox393-396D17, in the Col-0 background) (Gao et al., 2009) and bir1-2 (RATM11-5031-1, G in the No-0 background) T-DNA knockout lines were obtained from the ABRC, and the RIKEN
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Figure 10. Phosphorylation of BON1 and BIR1 by BAK1 in vitro. Affinity-purified recombinant proteins were incubated in the presence of [γ-32P]ATP. After SDS-PAGE, the reaction products were analyzed as indicated below. The top panel shows autoradiography; the bottom panel shows Coomassie Brilliant Blue staining. (a) Autophosphorylation of BIR1-GST Coomassie Brilliant Blue staining. shows autoradiography; the bottom panel shows ATP. After SDS-PAGE, the reaction products tomm panel). (b) No autophosphorylation activity of BON1 or phosphorylation of BON1 by BIR1 was observed. Purified BON1-GST proteins have two forms with different sizes as denoted with arrows. Shown is autoradiographed for 2 h (top panel); SDS-PAGE gel with Coomassie blue-stained BIR1-GST protein (bottom panel). (c) Phosphorylation of BON1 by BAK1 in vitro. The position of MBP protein is the same as upper band of BON1. Shown is autoradiographed for 24 h (top panel); SDS-PAGE gel with Coomassie blue-stained MBP-BAK1 and BON1-GST proteins (bottom panel). (d) Phosphorylation of BIR1 by BAK1 in vitro. Shown is autoradiographed for 2 h (top panel); SDS-PAGE gel with Coomassie blue-stained BIR1-GST protein and MBP-BAK1 proteins (bottom panel).

BioResource Center, respectively. The bir1-2 mutant contains a Ds transposon in the 5’-UTR of the BIR1 gene. Homozygous insertion mutants were identified by PCR with T-DNA left border (LB) primers and the gene-specific primers BIR1-p1 and BIR1-p2 (for bir1-1), and BIR1-p4, and BIR1-p5 (for bir1-2) listed in Table S1. The bir1-1/bir1-2 heterozygous plants were crossed with snc1-11 (Yang and Hua, 2004), bon1-1 (Hua et al., 2001) and three independent 35S::BON1 (Yang et al., 2007) transgenic lines to generate bir1-1 snc1-11, bir1-1 bon1-1 and bir1-1 35S::BON1 plants. Double mutant plants were identified in the F2 progenies by PCR.

The BIR1 cDNA was amplified by PCR using primers BIR1-p3 and BIR1-p2 and cloned into binary vector pSuper1300 (Yang et al., 2010) to generate pSuper::BIR1. Similarly, BIR1 cDNA lacking its stop codon was amplified using the BIR1-p3 and BIR1-p6 primers and fused with GFP in-frame in pGPTVII.GFP (Walter et al., 2004) to generate p35S::BIR1-GFP. Agrobacterium tumefaciens strain GV3101 carrying different constructs was used to transform bir1-1 or bon1-1 via floral dip transformation (Clough and Bent, 1998).

Yeast two hybrid assay

Yeast two-hybrid screening was performed using MATCHMAKEr GAL4 Two-Hybrid System 3 kit according to the supplier’s instruction (Clontech, http://www.clontech.com/). The A domain of BON1 fused with the DNA-binding domain of GAL4 in the yeast vector pGKT7 (Hua et al., 2001) was transformed into the yeast strain AH109 (Clontech). The resulting yeast cells were transformed with plasmid DNA derived from an Arabidopsis cDNA library obtained from ABRC (Stock No. CD4-10). Yeast transformants were screened on SC with glucose but lacking leucine, tryptophan, adenine, and histidine (SC/Leu/-Trp/-Ade/-His), followed by staining with β-galactosidase. Plasmid DNA was recovered from positive yeast colonies, transformed into Escherichia coli strain DH5α, and re-isolated for DNA sequencing.

To construct the vectors for yeast two hybrid assays, the BAK1-KD (amino acids 250–615) was PCR-amplified using its full-length cDNA as a template with primers BAK1-p1 and BAK1-p2. The PCR product was ligated into the pMD18-T vector (Takara, http://www.takara-bio.com/) and subcloned into the EcoRI/Xhol sites of pGBK7 (Clontech).

The BIR1 full-length cDNA was amplified by PCR using BIR1-p3 and BIR1-p6 primers. The BIR1-JKC fragment was amplified by PCR using BIR1-p9 and BIR1-p6 primers. The BIR1-KD fragment was amplified by PCR using BIR1-p13 and BIR1-p10 primers. The BIR1-CT fragment was amplified by PCR using BIR1-p11 and BIR1-p6 primers. All the PCR products were cloned into pGADT7 (Clontech).

Quantitative RT-PCR

Total RNA was isolated from 3-week-old plants with TriReagent (Sigma-Aldrich) followed by treatment with RNase-free DNase I (Promega, http://www.promega.com/) at 37°C for 1 h. The treated RNA samples (1 μg each) were used as templates for first-strand cDNA synthesis (Promega). Real-time PCR was performed using Applied Biosystems 7500 real-time PCR system (Applied Biosystems, http://www.appliedbiosystems.com/) with SYBR Premix Ex Taq (Takara). Relative expression levels were calculated as described (Huang et al., 2010b).

GUS assay

A 1.2 kb genomic fragment upstream of the BIR1 ATG start codon was amplified by PCR using the BIR1-p7 and BIR1-p8 primers. The amplified fragment was fused with the GUS reporter gene in the binary vector pZPGUS2 (Diener et al., 2000). Histochemical
detection of GUS activity was performed as described (Huang et al., 2010a).

**BiFC assays**

The BIR1 and BAK1 coding regions were cloned into pSPYCE-3SS (Walter et al., 2004) to form C-terminal fusions with YFP2, whereas the BON1 cDNA was cloned into pSPYN-E-3SS to generate an N-terminal fusion with YFPN. The plasmids were introduced into A. tumefaciens strain GV3101 and infiltrated into N. benthamiana according to the protocol described (Walter et al., 2004). For transient expression in protoplasts, plasmids of BON1-YFPN, BIR1-YFP2, BAK1-YFP2, and CLV1-YFP2 were co-transformed into the Arabidopsis protoplasts following the protocol described (Yoo et al., 2007).

The fluorescence of YFP in the transformed N. benthamiana leaves and protoplasts was visualized by confocal laser scanning microscope (LSM510, Carl Zeiss, http://www.zeiss.com/) 2–4 days after infiltration.

**Co-immunoprecipitation assay**

The coding sequences of BIR1, BAK1 and BON1 were fused translationally with the c-myc or haemagglutinin (HA) tags and cloned into the pSuper1300 (Yang et al., 2010) and pGreen0229 (Hellens et al., 2000), respectively. The plasmids were purified by CsCl gradient centrifugation and transformed into Arabidopsis mesophyll protoplasts (Yoo et al., 2007). After overnight incubation, the protoplasts were lysed with extracting buffer (50 mM HEPES-KOH (pH 7.5), 1 mM DTT, 10 mM MgCl2, 150 mM NaCl, 1 mM PMSF and 1× protease inhibitor (PI) cocktail (Sigma-Aldrich)) and centrifuged. The protein extracts were then incubated with anti-myc agarose conjugate (Sigma-Aldrich) at 4°C for 2 h. After washing five times with extraction buffer, the co-immunoprecipitation products were detected by anti-HA antibody. Anti-myc (Sigma-Aldrich) and anti-HA (Sigma-Aldrich) antibodies were used at 1:5000 and 1:1000 dilutions respectively, and chemiluminescence signals were detected by Western Blotting Luminol Reagent (Santa Cruz, http://www.scbt.com)/.

**Protein expression**

BIR1-JKC and the BON1-A were amplified and cloned into pGEKT-1 with the GST-tag (GE healthcare, http://www.gelifesciences.com/). BAK1-KD was cloned into pMal-c2::His with His and MBP-tag derived from pMal-c2 (New England Biolab, http://www.neb.com/). To generate mutation form of BIR1 and BAK1, site-directed mutagenesis was performed using the QuickChange Site-Directed Mutagenesis kit (Stratagene, http://www.genomics.agilent.com/) and mutations were confirmed by sequence analysis. The primers used for mutagenesis are listed in Table S1. All these plasmids were transformed into E. coli strain BL21 (DE3). The recombiant proteins were purified with glutathione Sepharose 4B or Ni Sepharose 6 Fast Flow (GE healthcare) according to the manufacturers’ instructions.

**Pull-down assay**

For the pull-down assay, GST and BIR1-JKC-GST fusion proteins were kept immobilized on glutathione Sepharose 4B beads (GE). GST and BIR1-JKC-GST-bound beads were re-suspended in blocking buffer (400 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.05–0.1% NP-40, 0.5% non-fat milk) and gently rotated at 4°C for 1 h. Next, the beads were centrifuged and the blocking buffer was discarded. The MBP and BON1A-MBP fusion proteins were added into the GST and BIR1-JKC-GST-bound beads, respectively. The resulting solutions were re-suspended in binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.05–0.1% NP-40) and incubated at 4°C for 1 h. Subsequently, the beads were washed with wash buffer (400 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, 0.05–0.1% NP-40) at least five times. The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the MBP and BON1A-MBP signals were detected by western blotting with an anti-His antibody (MBL, http://www.mblint.com/).

**Protein kinase assay**

For kinase assay, 2 μg of fusion proteins (except for 6 μg of GST-BON1 protein) were incubated in kinase reaction buffer containing 50 mM HEPES-KOH (pH 7.5), 1 mM DTT, 10 mM MgCl2, 10 mM MnCl2, 50 mM ATP, and 1 μCi of [γ-32P]ATP in a final volume of 20 μl. The reaction was incubated for 30 min at 30°C, terminated by adding equal volume of 2× SDS sample buffer, and then heated at 95°C for 5 min. The samples were then separated on a 12% (w/v) SDS-PAGE gel, and autoradiographed.

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**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Alignment of the deduced amino acid sequences of BIR1 and its homologs from Arabidopsis.

**Figure S2.** BiFC analysis of BIR1 and BON1 interactions in N. benthamiana leaves.

**Figure S3.** Comparison of fresh weights of plants.

**Table S1.** Gene-specific primers used in this study.

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**REFERENCES**


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