Multiple *R*-Like Genes Are Negatively Regulated by *BON1* and *BON3* in *Arabidopsis*

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The *Arabidopsis thaliana* genome contains more than 200 rapidly evolved resistance (*R*)-like genes coding for nucleotide binding leucine-rich repeat (NB-LRR) and their related proteins. A dozen of them are shown to play key roles in plant responses to biotic attacks, and they need to be repressed in the absence of biotic stresses to prevent activation of defense responses that are usually detrimental to plant growth and development. Here, we show that the *Arabidopsis BON1* and *BON3* genes, two members of the evolutionarily conserved copine, are negative regulators of several *R*-like genes. At least four such genes of the Toll-interleukin-1 receptor-like (TIR)-NB-LRR or TIR-NB type were identified through their activities in triggering cell death in the absence of the *BON1* and *BON3* function and their natural variations between two *Arabidopsis* accessions, Col-0 and Ws-2. These so-named lesion cell death (*LCD*) genes contribute quantitatively to the phenotypes of enhanced defense response and cell death in the *bon1bon3* mutant. Further, their activation in the *bon1bon3* mutants appears to be through different regulatory modes, and *BON1* and *BON3* may repress the transcript accumulation or protein activities of these *R*-like genes.

Plants have evolved sophisticated adaptive mechanisms to perceive and respond to various pathogen attacks (Chisholm et al. 2006; Dangl and Jones 2001; Jones and Dangl 2006). In addition to general basal defenses, plants use disease resistance (*R*) genes for race-specific defenses. Allele-specific genetic interaction between a pathogen avirulence (*Avr*) gene and a plant *R* gene triggers hypersensitive response (HR), a form of programmed cell death, which limits pathogen growth rapidly and efficiently (Scheel 1998). Despite the wide range of pathogen taxa and their pathogenicity effector molecules, *R* genes cloned so far encode mostly five classes of proteins (Martin et al. 2003). The biggest class of *R* genes encodes proteins with nucleotide binding leucine-rich repeat (NB-LRR) domains. According to bioinformatics analysis combined with experimental validation, there are approximately 159 NB-LRR-encoding *R*-like genes and 58 *R*-like genes without LRR in the *Arabidopsis*

thaliana genome (Meyers et al. 2003). According to their amino-terminal structure, this class can be divided into two subclasses, with either a Toll-interleukin (IL)-1 receptor like (TIR) domain or a coiled-coil (CC) domain. The TIR type of *R* proteins appears to have undergone large expansions and out-numbered the CC type in *Arabidopsis*. *R* proteins are thought to act as molecular switches of plant defense and the mode of their activation and signaling have been inferred from mutational and structure-function analyses (DeYoung and Innes 2006; Takken et al. 2006). Its specificity is likely conferred by the amino-terminal domain and possibly the LRR domain as well. The LRR domain also acts as a regulatory domain for the NB domain that binds and hydrolyzes ATP and acts as a switch for *R* protein activity.

The NB-LRR genes are thought to be real *R* genes serving to perceive various pathogens, although only a small proportion of them have known corresponding *Avr* genes or pathogens they detect (Tan et al. 2007). For simplicity, we will refer to NB-LRR genes as *R* genes or *R*-like genes. These genes exist either in single *R*-gene loci or in clusters of *R* genes in *Arabidopsis*, and they often have a high degree of genetic variation among different ecotypes. Many *R*-gene clusters comprise several copies of homologous genes probably arising from a single progenitor, whereas other clusters contain *R* genes from different phylogenetic clades sometimes interspersed with unrelated genes. The NB-LRR loci are not evenly distributed in the *Arabidopsis* genome: both chromosome 1 and 5 contain super clusters, whereas chromosome 2 and 3 are relatively deficient in *R*-gene clusters (Meyers et al. 2003).

The evolution and regulation of *R* genes are likely shaped by the interaction between plants and pathogens as well as balances between plant defense and plant fitness. Resistance responses can impose fitness costs under the pressure of pathogen attack and even in the absence of pathogens (Korves and Bergelson 2004; Tian et al. 2003). Many mutants with constitutive activation of *R*-gene-mediated resistance exhibit growth defects with spontaneous lesions (cell death) (Shirano et al. 2002; Yang and Hua 2004). Therefore, it is necessary for plants to have a tight control on *R* gene expression and activity to maximize plant growth and fitness.

R genes are subject to complex regulations at multiple levels. First, the activities of *R* proteins are fine tuned. Direct interactions between some *R* and *Avr* protein pairs have been observed (Bent and Mackey 2007). However, attempts to detect a physical interaction between an *R* and its corresponding *Avr* protein have not been successful for most of the *R* proteins. Rather, some plant host proteins were found to act as mediators of *Avr*–*R* interaction (Belkadir et al. 2004). In this scenario of “guard hypothesis” (Van der Biezen and Jones 1998), the pathogen *Avr* protein interacts with and modifies a host protein or proteins, and the corresponding *R* protein recog-

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nizes (“guards”) the modification of the host factor to initiate defense responses. For example, the RIN4 protein is guarded by two R proteins, RPS2 and RPM1, and it negatively regulates their function (Axtell and Staskawicz 2003; Mackey et al. 2002, 2003). Second, the transcription of *R* genes could be directly regulated by Avr proteins. For instance, the *R* gene Xa27 is activated transcriptionally by the AvrXa27 protein that contains a transcription activation domain (Gu et al. 2005). Third, *R* genes are subject to epigenetic regulation. An epi-allele of an *R*-like gene *SNCI* (*suppressor of npr1, constitutive 1*) named *bal* overexpresses *SNCI* and consequently activates salicylic acid (SA)-dependent defense responses (Stokes et al. 2002). Genes in the *RPP5* cluster are found to be co-regulated, probably through gene-silencing machinery (Yi and Richards 2008). Study of autoactivation of *SNCI* as a transgene indicates that this *R*-like gene is regulated at the transcript level by different factors, including chromatin structures and the *SNCI* gene itself (Li et al. 2007).

BON1 (*BONZAI1*) is a negative regulator of the *R*-like gene *SNCI*. The loss of *BON1* function leads to a temperature-dependent growth defect due to an activation of *SNCI* (Hua et al. 2001; Yang and Hua 2004). The *Arabidopsis BON1* gene belongs to the evolutionarily conserved copine family found in protozoa, plants, nematodes, and mammals (Creutz et al. 1998); however, the biological and biochemical function of this family is not well understood. *BON1* is the first copine gene whose function was revealed genetically. *BON1* has two homologs in *Arabidopsis*: *BON2* and *BON3* (Yang et al. 2006b). Characterization of the single and multiple mutant combinations of the *BON1* family showed that the three copines have overlapping functions essential for the viability of plants (Yang et al. 2006b). Although the *bon1-1* single mutant shows a temperature-dependent dwarf phenotype, the *bon1-1bon2-2* and *bon1-1bon3-3* double mutants and the *bon1-1bon2-2bon3-3* triple mutant are seedling lethal. Further upregulation of defense-related genes was detected in these mutants compared with the *bon1-1* single mutant. Mutations in *EDS1* or *PAD4*, two key regulators in defense response and cell death (Wiermer et al. 2005), rescue totally or partially the growth and cell death phenotype in these mutants. These results indicate that the *BON1*, *BON2*, and *BON3* genes act as negative regulators of defense responses and cell death.

To further reveal the function of the *BON1* family, we sought to isolate genes mediating the combination mutant phenotypes of the *BON* genes by taking advantage of the phenotypic differences between the *bon1bon3* double mutants in two accessions. The *bon1bon3* double mutant in the Columbia-0 (Col) accession background is seedling lethal whereas it has a wild-type appearance in the Wassilewskija-2 (Ws) accession background. This variation indicates the existence of natural modifiers of *bon1bon3*. The *R*-like gene *SNCI* is a natural modifier of the *bon1* single mutant (Yang and Hua 2004). Its presence in Col leads to the mutant growth phenotype of *bon1-1*, and the lack of *SNCI* in Ws leads to the wild-type-like phenotype of *bon1-2*. However, *SNCI* is not the only natural modifier of *bon1bon3* because the loss-of-function mutation *snc1-11* weakens but does not totally rescue the mutant phenotype of *bon1-1bon3-3* in Col, and the *bon1-1bon3-3snc1-11* triple mutant eventually dies without bolting (Yang and Hua 2004). We hypothesize that additional modifiers of *bon1bon3* exist, and we name them *lesion cell death* genes (*LCD*) because they cause cell death in the Col *bon1bon3* mutant. *LCD* are likely target genes regulated by *BON1* and *BON3*, and they are responsible for the phenotypic variations between the *bon1bon3* phenotypes in the Col and Ws accessions.

Here, we report the rough mapping of *LCD* with recombinant inbred lines (RIL) of *bon1bon3* and the identification of at least

four *LCD* through genetic means. These *LCD* all encode *R*-like genes that were not previously characterized, thus revealing new regulations of *R*-like genes. These genes are normally kept inactive by *BON1* and *BON3* at either the transcript or protein activity level. The derepression of these genes in *bon1bon3* quantitatively contributes to the lethality in the double mutant. These findings allow further mechanistic studies of regulation of *R*-like genes and the function of the copine genes.

RESULTS

Accession-dependent cell death phenotype of *bon1bon3* mutant is controlled by multiple loci.

To identify the *BON1* and *BON3* target gene *LCD*, we constructed a mapping population by crossing *bon1-1bon3-3snc1-11* (in Col) to *bon1-2bon3-1* (in Ws). The F1 plants did not exhibit obvious defects under normal growth conditions (22°C, 50 to 70% relative humidity, and constant light), and the F2 plants exhibited cell death phenotypes to a different extent. The segregation of the cell death phenotype in the F2 population was variable from one planting to another, and the ratio of plants with and without cell death phenotypes often did not fit that of a trait controlled by a single locus or two loci, indicating the existence of multiple *LCD*.

Therefore, we generated RIL between *bon1-1bon3-3snc1-11* (Col) and *bon1-2bon3-1* (Ws) to assist the mapping of multiple genes and to minimize variability in phenotypic scoring. In total, 115 F2 plants were raised at 28°C to rescue plants that

Fig. 1. Phenotypes of *bon1bon3* recombinant inbred lines (RIL). **A**, Classification of cell death phenotypes in RIL. Shown are representative RIL lines from each category grown for 3 weeks (upper panel) and 5 weeks (lower panel) at 22°C. Plants of level 1 (L1) are largely wild-type in appearance. The L2 plants are overall wild-type in appearance but with minor cell death. The L3 plants show severe cell death but can bolt. The L4 and L5 plants can never bolt due to severe cell death, and the L5 plants were smaller than the L4 plants. Inserts are blown-out images for L3, L4, and L5 plants. **B**, Staining of leaf tissues by 3,3'-diaminobenzidine (DAB) to show the accumulation of H₂O₂. RIL with more severe cell death have stronger DAB staining, indicating a higher H₂O₂ accumulation. L1 through L5 = level 1 through level 5.

would die at 22°C, and they were propagated by single-seed descendant at 28°C for four to five generations to create RIL. When grown at 22°C, different RIL displayed various degrees of cell death (Fig. 1A). To quantify this phenotype or trait, we classified cell death semiquantitatively into five levels (L1 through L5) by their visual characteristics at 22°C. L1 plants had a wild-type appearance without any visible cell death. The L2 plants had minor cell death on leaf tips and sometimes sepal tips while the whole plants were largely wild type in appearance. The L3 plants were smaller than the wild type and had extensive cell death on rosette leaves, but they could flower and set seed like the wild type. Both the L4 and L5 plants had very severe cell death on leaves and could never bolt. The L5 plants were much smaller than the L4 plants, and they had an even more severe growth defect phenotype than the Col parent. All the RIL were scored for their growth phenotypes independently at two consecutive selfing generations (fifth and sixth or sixth and seventh) at 22°C, and their consistent classification levels indicate the reproducibility of the classification system.

We determined whether the visual classification of RIL also reflects their molecular and physiological features such as the accumulation of reactive oxygen species (ROS) and the activation of defense responses. To this end, three RIL from each of the five levels were randomly selected for analyses. First, we

used 3,3'-diaminobenzidine (DAB), a substance reacting with H₂O₂ to produce brown coloration, to analyze the accumulation of H₂O₂ in RIL because cell death is usually accompanied by an overaccumulation of ROS such as H₂O₂. L1 and L2 plants had the weakest staining of DAB, L3 and L4 had stronger staining, and L5 plants showed the strongest staining (Fig. 1B). Thus, a higher accumulation of H₂O₂ is largely correlated with an increase in visual cell death. Second, we analyzed by RNA blots the expression of *PR1*, a molecular marker for SA-mediated defense response. *PR1* was upregulated in all the *bon1-1*, *bon1-1bon3-3*, and *bon1-1bon3-3snc1-11* mutants compared with the wild-type Col; and it was weakly induced in *bon1-2* and *bon1-2bon3-1* compared with the wild-type Ws (Fig. 2A). For the two parental lines for RIL, the *PR1* expression was higher in *bon1-1bon3-3snc1-11* than in *bon1-2bon3-1*, consistent with their morphological phenotypes. In all intercrossed RIL, *PR1* was induced compared with the wild-type Col (Fig. 2A). Although there were variations of *PR1* expression within the same level group, its expression level was largely but not strictly correlated with the severity of the cell death phenotype. The L3 and L4 plants had at least three times more *PR1* expression than the L1 and L2 plants, and the L5 plants had the highest expression of all. Third, we assayed disease resistance to the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in representative RIL. Plants were grown under a photoperiod of 12 h of light and 12 h of darkness, where the morphological differences among different LCD levels were substantially reduced. Young seedlings, approximately 2 weeks old, were dip inoculated with DC3000 and leaf tissues were collected at 1 h (day 0) and 3 days (day 3) after inoculation. The amount of bacteria in these tissues was assayed by grinding the leaf tissues and plating serial dilutions of extracts on solid media. Three days after inoculation of the pathogen, L4 and L5 plants supported much less bacterial growth than the L1, L2, and L3 plants. *P. syringae* pv. *tomato* DC3000 grew to 2.5×10^5 and 3.2×10^5 CFU/mg in the wild-type Col and Ws, respectively, whereas it grew to 2×10^5 , 3×10^5 , 2×10^5 , 2.5×10^4 , and 5×10^3 CFU/mg in L1 through L5, plants respectively (Fig. 2B). Thus, the degree of disease resistance was correlated with the level of *PR1* gene expression among RIL. These analyses indicate that defense responses and cell death are triggered to a different extent in these *bon1bon3* RIL and that the visual classification largely reflects the molecular and physiological features of the plants.

Fig. 2. Activation of defense responses in recombinant inbred lines (RIL). **A**, *PR1* gene expression in RIL. Shown is the RNA blot analysis of *PR1* in representative RIL compared with the wild type and the *bon* mutants. The expression level of *PR1* is largely correlated with the cell death level. The L3, L4, and L5 plants with more severe cell death have a higher expression of *PR1* than the L1 and L2 plants with less severe cell death. **B**, Resistance to virulent pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in RIL. Shown is the bacterial growth in representative RIL at 1 h (day 0) and 3 days (day 3) after *P. syringae* pv. *tomato* DC3000 was inoculated by dipping. Enhanced pathogen resistance is correlated with a higher *PR1* expression.

Rough mapping of LCD.

To map the LCD, we selected 29 molecular markers with polymorphisms between Col and Ws, and they distribute quite evenly among the five chromosomes (Fig. 3A). Of these, 24 were existing markers in the TAIR database and the remaining five (TIR, JH2, JH3, JH7, and JH9) were new markers (Supplementary Table 1). All 115 of the RIL were subject to genotyping with these 29 markers. Phenotyping data was confirmed by growing out each RIL twice and consistent classification was obtained. Both the genotyping (Col and Ws) and phenotyping (semiquantification of 1 through 5 for L1 through L5) data were input into the program QTL cartographer. Composite interval mapping was used for quantitative trait loci (QTL) analysis and a 1,000× permutation test was analyzed. With a significance level of 0.05, the log-likelihood ratio (LR) value of this analysis was 12.58, very close to the default value 11.5. Therefore, the default value was used for later analysis. The data output indicated that there was no significant linkage on chromosome 2 and 4. Some noises were seen on chromosome 5, especially at the end of chromosome 5. This may be due to a high density of minor QTL on chromosome 5 or the end-of-chromosome effect of the software.

The data output also showed distinct peaks on chromosomes 1 and 3 (Fig. 3B). With a $\log_{10}$ likelihood ratio (LOD) cutoff of 1, at least five QTL (named QTL1 through QTL5) were detected for *LCD*. QTL1 and 2 on chromosome 1 and QTL4 and 5 on chromosome 3 showed a positive contribution from Col while QTL3 on the bottom of chromosome 1 showed a positive contribution from Ws. The extreme severe phenotype in the L5 plants is likely contributed by QTL3 from Ws in combination with other QTL from Col. These five QTL each contributed 7, 12, 12, 4, and 12% of the cell death phenotype, respectively (Fig. 3B). The LOD scores for these associations were not very high, likely due to the low marker density and the semiquantitative classification of the trait. However, these QTL are most likely not false positives for the following reasons. First, the associations were robust. Identical peaks were identified when the classification was changed from five levels to two levels; that is, when L1 and L2 were combined into one level and L3 to L5 into another (data not shown). Second, a correlation between the genotype at these QTL and the mutant phenotype was observed in the *bon1-bon3* RIL. Among the 47 RIL with a cell death level at L3 through L5, 43 lines had at least two positive *LCD* QTL at these five identified loci. A stronger association was observed for the L4 and L5 lines that contained mostly positive *LCD* QTL. Third, a student *t* test with multivariate on the phenotype and QTL genotype of RIL showed that most of the QTL-linked markers are correlated with the phenotype strongly (data not shown). These analyses support the QTL analysis result.

Identification of *LCD*.

We noticed that each of these QTL is located close to one or more clustered NB-LRR-type *R* genes on the chromosomes. This could be a coincidence because *R* genes are numerous in the *Arabidopsis* genome. Alternatively, this might suggest that *LCD* are some of these *R* genes. The latter hypothesis was supported by a new marker generated within the cluster of *R* genes close to QTL1. It was found to be more closely associated with *LCD* than the original marker used (data not shown).

We set out to test the hypothesis that some of the *R*-like genes in these clusters are *LCD*. The lethality in *bon1-bon3-3* (Col) is likely due to an activation of *LCD* (*R* genes) in Col but not in Ws. The loss-of-function mutants of *LCD* might partially suppress the cell death phenotype of *bon1-bon3-3*. The rescue of lethality by *eds1* and *pad4* is consistent with this hypothesis. We focused on QTL with positive contributions from the Col alleles because a large collection of transfer-DNA (T-DNA) induced mutants is available in Col (Alonso et al. 2003). In all, 10 annotated *R*-like genes (Meyers et al. 2003) reside within two adjacent markers flanking the one closest to each of

QTL1, QTL2, QTL4, and QTL5; these 10 genes are referred to as *LCD* candidate (*LCC*)1 to *LCC*10 (Table 1). QTL1 is close to a single gene, *LCC1*, and a gene cluster consisting of *LCC2* to *LCC4*. QTL2, QTL 4, and QTL 5 are each close to gene clusters consisting of *LCC5* and *LCC6*, *LCC7* and *LCC8*, and *LCC9* and 10, respectively.

Fig. 3. Mapping of lesion cell death (*LCD*) genes. **A**, Distributions of molecular markers for *LCD* mapping on five chromosomes. Marker names are on the right, and their positions shown on the left are expressed as centimorgans from the top of the chromosome. Markers labeled with asterisks are associated with quantitative trait loci (QTL). **B**, Output data from QTL Cartographer. The upper panel shows the peaks linked with the cell death phenotype. The five QTL identified are numbered 1 to 5. The middle panel shows the additive genetic effects. QTL3 is linked to Wassilewskija-2 and all other QTL are linked to Columbia-0. The bottom panel shows the coefficient of determination R^2 value. LOD = $\log_{10}$ likelihood ratio.

Table 1. Lesion cell death candidate genes (*LCC*)^a

Name	Gene locus	QTL	<i>R</i> gene type ^b	T-DNA line	Insertion site
<i>LCC1</i>	At1g15890	1	CNL-B	<i>lcc1-1</i> :SALK_055219	Exon 1
<i>LCC2</i>	At1g17600	1	TNL-D	<i>lcc2-1</i> :SALK_069931	Exon 4
<i>LCC3</i>	At1g17610	1	TN	<i>lcc3-1</i> :SAIL_861_D11	3' untranslated region
<i>LCC4</i>	At1g17615	1	TN	<i>lcc4-1</i> :SALK_062069	Exon 2
<i>LCC5</i>	At1g27170	2	TNL-C	<i>lcc5-1</i> :SALK_104254	Exon 1
<i>LCC6</i>	At1g27180	2	TNL-C	<i>lcc6-3</i> :SALK_132462	Exon 4
<i>LCC7</i>	At3g25510	4	TNL-G	<i>lcc7-3</i> :SALK_032836	Exon 3
<i>LCC8</i>	At3g25515	4	TNL-G	<i>lcc8-1(lcc7-4)</i> :SALK_119130	Exon 6 of <i>LCC7</i> ; exon 2 of <i>LCC8</i>
<i>LCC9</i>	At3g51560	5	TNL-A	<i>lcc9-1</i> :SALK_094434	Exon 1
<i>LCC10</i>	At3g51570	5	TNL-B	<i>lcc10-1</i> :SALK_057226	Exon 4
<i>SNC1</i>	At4g16890	N/A	TNL-E	<i>snc1-11</i> :SALK_047058	Exon 1

^a Shown are the gene identification numbers, linked quantitative trait loci (QTL), resistance (R) protein types, transfer-DNA (T-DNA) insertion lines, and the insertion sites for the *LCC* genes.

^b R protein type: CNL = coiled-coil nucleotide binding leucine-rich repeat (CC-NB-LRR), TNL = Toll-interleukin-1 receptor-like (TIR)-NB-LRR, and TN = TIR-NB. The letter after dash represents the evolutionary group as described (Meyers et al. 2003); N/A = not applicable.

In total, 10 T-DNA insertion lines in Col were obtained from the *Arabidopsis* Biological Resource Center (ABRC) for the genes *LCC1* through *LCC10*, and they are named *lcc1-1*, *lcc2-1*, *lcc3-1*, *lcc4-1*, *lcc5-1*, *lcc6-3*, *lcc7-3*, *lcc8-1*, *lcc9-1*, and *lcc10-1* (Table 1). T-DNA insertions were confirmed to reside in exons of these genes except for *LCC3*, where the T-DNA was in the 3' untranslated region (UTR) (Table 1) and, therefore, all these lines (excluding *lcc3-1*) are likely to be knockout mutants of the respective genes. *LCC8* was annotated as a gene with a coding region partially overlapping with that of *LCC7*, and the mutant *lcc8-1* has a T-DNA inserted in the overlapping part of *LCC7* and *LCC8*. Because *LCC8* is no longer considered to be a real gene in the new genome version, we renamed the *lcc8-1* mutant as *lcc7-4*. Homozygous lines of these *lcc* mutants were selected by polymerase chain reaction (PCR)-based genotyping, and none of them exhibited obvious growth defects.

To assay their effects on *bon1* and *bon1bon3* in Col, these *lcc* mutants were crossed to *bon1-1* and *bon1-1bon3-3snc1-11*, respectively. Double mutants between *lcc* and *bon1-1* were isolated, and all 10 of the *lccbon1-1* mutants looked like *bon1-1* (data not shown), indicating that these *LCC* genes do not have a major effect on the *bon1-1* single mutant. Six of the *lccbon1-1bon3-3* triple mutants were also identified. Although the *lcc1* and *lcc5* mutants did not appear to affect *bon1-1bon3-3* (data not shown), *lcc2*, *lcc4*, *lcc6*, and *lcc7* partially rescued the *bon1-1bon3-3* mutant phenotype (Fig. 4A). The *bon1-1bon3-3* double mutants had arrested growth after the emergence of the two cotyledons and died without having true leaves at 22°C. In contrast, these four *lcc* triple mutants produced at least two true leaves and lived longer than the *bon1-*

lbon3-3 double mutant. None of these *lcc* mutants could totally rescue the lethality phenotype, and they eventually died before bolting. Therefore, *LCC2*, *LCC4*, *LCC6*, and *LCC7* are real *LCD* mediating the growth defects and likely the disease resistance phenotype in *bon1bon3* and, therefore, they are renamed as *LCD2*, *LCD4*, *LCD6*, and *LCD7*. *LCC1* and *LCC5* are probably not *LCD* because their loss-of-function mutants do not appear to have an obvious effect on the *bon1bon3* phenotype (data not shown). It was not determined whether *LCC3*, *LCC9*, or *LCC10* are *LCD* because their triple mutants with *bon1-1bon3-3* are yet to be characterized.

In parallel, we used virus-induced gene silencing (VIGS) (Ruiz et al. 1998) as a secondary approach to determine whether knocking down the activities of any of the *LCC* genes can partially rescue the cell death phenotype of *bon1-1bon3-3*. Nine *LCC* genes (*LCC1*, *LCC2*, *LCC3*, *LCC4*, *LCC5*, *LCC6*, *LCC7*, *LCC9*, and *LCC10*) were each cloned into the VIGS vector (Burch-Smith et al. 2006). The *bon1-1bon3-3* plants grown at 28°C were subject to VIGS treatment and their phenotypes were analyzed after they were moved to 22°C. The *bon1-1bon3-3* died a week after moving to 22°C without any green inflorescence tissues (data not shown). In contrast, at least 30% (a total of nine each) of VIGS-treated plants by the two control genes *PAD4* and *SNC1* had green inflorescences (Fig. 4B). These two genes have been demonstrated to mediate the cell death phenotype of *bon1-1bon3-3* (Yang et al. 2006b), and their cell death suppression effects indicate the effectiveness of VIGS in this system. Although *bon1-1bon3-3snc1-11* is seedling lethal and *bon1-1bon3-3pad4-1* is wild type, the *SNC1* knockdown rescued the *bon1bon3* mutant as well as the *PAD4* knockdown in this VIGS system. This is probably due to the early rescue of *bon1bon3* by growth at 28°C for 2 weeks. More than 20% of plants VIGS treated by the *LCC2*, *LCC4*, *LCC7*, and *LCC9* genes had green inflorescences similar to those of *PAD4* and *SNC1* (Fig. 4B), while all of the plants treated by *LCC1*, *LCC3*, *LCC5*, *LCC6*, and *LCC10* exhibited the same death phenotype as the no-VIGS control (Fig. 4B and data not shown). This data is largely consistent with results from using T-DNA insertion mutants. It corroborated our conclusion that *LCC2*, *LCC4*, and *LCC7* are real *LCD* while *LCC1* and *LCC5* are probably not. However, VIGS does not always knock down the target genes below its functional level, which would explain the failure to suppress cell death by VIGS of *LCC6*. In addition, VIGS could target homologous genes for degradation and, thus, sometimes lacks specificity. Therefore, the identity of *LCC9* as a real *LCD* awaits confirmation by using the T-DNA mutant.

Characteristics of the LCD proteins.

None of these *R*-like genes had been previously identified or characterized functionally. The identification of these genes as *LCD* indicates that they are functional and possess cell-death-inducing activity similar to real *R* genes. We used the pfam program to predict the domain structures of identified LCD proteins, including *LCD2*, *LCD4*, *LCD6*, *LCD7*, *LCC8*, and *SNC1* (Fig. 5A). The common feature of these *LCD* is that all of them contain one TIR domain (*LCD2*, *LCD4*, *LCD7*, *LCC8*, and *SNC1*) or two TIR domains (*LCD6*). However, these *LCD* do not seem to belong to a specific subgroup of the TIR-NB-LRR type of *R* proteins. According to the classification based on sequence homologies (Meyers et al. 2003), *LCD2* belongs to the TNL-D type, *LCD6* to the TNL-C type, *LCD7* to the TNL-G type, and *SNC1* to the TNL-E type (Table 1). The number of LRR in these proteins also varies greatly, ranging from 5 to 21. Thus, the *LCD* proteins do not appear to share high protein sequence homology except for containing the TIR domain.

Fig. 4. Identification of lesion cell death genes. **A**, Phenotypes of *bon1-1bon3-3lcc* plants. Plants were grown at 22°C for 3 weeks. The *bon1-1bon3-3* mutant does not develop true leaves. Mutations in *lesion cell death* candidate (*LCC*) genes *LCC2*, *LCC4*, *LCC6*, and *LCC7* partially rescue the *bon1-1bon3-3* cell death phenotype. Note that the triple mutants all developed true leaves and died later than the *bon1-1bon3-3* mutant. **B**, Phenotypes of *bon1-1bon3-3* with *LCC* genes knocked down by virus-induced gene silencing (VIGS). The *bon1-1bon3-3* plants were grown at 28°C for 2 weeks and VIGS was performed to silence the *LCC* genes and the control *PAD4* and *SNC1* genes, respectively. Plants were moved to 22°C 3 days after VIGS treatment and shown are plants 1 week after growth at 22°C. Note that *LCC2*, *LCC4*, *LCC7*, and *LCC9* gave rescued plants with green inflorescences, similarly to *PAD4* and *SNC1*, whereas *LCC1* and *LCC10* as well as *LCC3*, *LCC5*, and *LCC6* (not shown) had dead inflorescences.

Expression of LCD.

The identification of *LCD* allowed us to analyze whether the target *R*-like genes are always upregulated at the transcript level in the *bon1bon3* mutant. All these *LCD* have very low expressions and could not be detected by RNA blot analysis except for *LCD2* (data not shown). Semiquantitative reverse-transcription (RT)-PCR showed that transcripts of *LCD2*, *LCD4*, and *LCD7* but not *LCD6* were upregulated in *bon1-1*, *bon1-1bon3-3*, and *bon1-1bon3-3snc1-11* mutants compared with the wild-type Col (Fig. 5B). Thus, *LCD* are not always regulated at the transcript level by *BON1* and *BON3*. *LCD4* and *LCD7* appeared to have a lower expression in *bon1-1bon3-3* and *bon1-1bon3-3snc1-11* than in *bon1-1* (Fig. 5B); however, this should be taken with caution because the *bon1-1bon3-3* and *bon1-1bon3-3snc1-11* plants had more extensive cell death than *bon1-1* even in the very young tissues analyzed. Some genes, probably including those of *LCD*, might be more susceptible to cell death and, thus, have reduced transcripts in tissues undergoing cell death. The *LCD* also do not appear to be regulated similarly at the transcript level by other factors such as SA (data not shown). Furthermore, no common *cis*-regulatory motifs were identified in the 1.5-kb promoter regions of these *LCD* with a *cis*-element finding program (data not shown).

Natural variations of LCD.

We selected *LCD2* and *LCD4* to represent the TIR-NB-LRR type and the TIR-NB type of *R* genes to investigate the nature of *LCD* variations between the Col and Ws alleles. The coding and the promoter regions of the two genes in Ws were sequenced and compared with those in Col. For *LCD2*, five nucleotide substitutions were found in the coding region of the

Ws allele. Two of them cause codon changes, with valine substituting alanine 905 and serine substituting asparagine 950. Both changes occur in the LRR region and they might account for an alteration in function or activity of the Ws *LCD2*. For *LCD4*, two nucleotide substitutions were found in the coding region of the Ws allele but neither of them caused amino acid residue changes or affected intron splicing pattern according to the prediction by NetGene 2. Three alterations were found in the promoter region of the Ws allele: C to T at -352 bp, T to A at -698 bp, and A to T at -1,205 bp relative to the translation start site. The expression of *LCD4* could not be detected by RT-PCR in the wild-type Col or Ws plants. *LCD4* could be amplified from *bon1-1* (Col) but not from *bon1-2* (Ws) (Fig. 5C). This suggests either a difference in RNA expression regulation of *LCD4* between two accessions or a lack of defense response activation even in the Ws double mutant. In any case, the comparison of these two *LCD* genes between Col and Ws already suggests that natural variations in *LCD* could result from polymorphisms in the coding regions as well as the regulatory regions.

Expression of the Col *LCD4* but not *LCD2* leads to growth defects.

The Ws *LCD* presumably are not expressed or are expressed but not activated in the Ws *bon* mutants. We hypothesize that cell death phenotype might occur in the Ws *bon1bon3* mutant if the Col *LCD* gene is expressed or activated. As a first step to test this, we cloned the Col genomic fragments of *LCD2* and *LCD4* in a binary vector and transformed them into Ws and Col. In total, 39 and 35 lines carrying the *LCD2* transgene were generated in Col and Ws, respectively. None of the *LCD2* transgenics exhibited an obvious mutant growth phenotype (Fig. 6). The Col and Ws *LCD2* proteins differ in their amino acid sequences and obtaining a Col *LCD2* in the Ws plants is not sufficient to cause a *bon1-1* like phenotype. This suggests a regulation of *LCD2* by *BON1* and *BON3* at the protein activity level, which needs to be tested by introducing the Col *LCD2* into *bon1bon3* double mutant in Ws.

In contrast, most of the *LCD4* transgenic lines in Ws (47 of 67) exhibited a *bon1-1*-like dwarf phenotype (Fig. 6). Most of the *LCD4* transgenic lines in Col (27 of 37) also exhibited a weak *bon1-1*-like phenotype with slightly smaller, curly leaves and water-soaked appearance (Fig. 6). This indicates that *LCD4* can induce growth defects when it is sufficiently expressed. *LCD4* has a very low expression in the wild type (not detectable by our RT-PCR), and the *LCD4* as a transgene likely has a higher expression level than the endogenous gene, similar to *SNC1* (Li et al. 2007). This higher expression of the *LCD4* transgene probably is sufficient to induce a mutant phe-

Fig. 5. Characterization of lesion cell death (*LCD*) genes. **A**, Domain structures of the *LCD* and *SNC1* proteins. The starting and ending residues of each domain are indicated. light boxes mark the Toll-interleukin-1 receptor-like domain, dark boxes mark nucleotide binding domain, and ovals mark the leucine-rich repeat domain. Lines mark the linker region that does not belong to any of the three domains. **B**, *LCD* RNA expression in different mutants. Shown is the semi-quantitative reverse-transcription polymerase chain reaction (RT-PCR) of *LCD* candidate genes in Columbia-0 (Col), *bon1-1*, *bon3-3*, *bon1-1bon3-3* and *bon1-1bon3-3snc1-11*. The tubulin gene is used as a control. **C**, Expression of *LCD4* in Col and Wassileskija-2 (Ws) accessions. Shown is the RT-PCR of *LCD4* in Col, *bon1-1*, Ws, and *bon1-2*. The RNA expression of *LCD4* cannot be detected by semiquantitative RT-PCR in wild-type or *bon* mutants in Ws. The tubulin gene is used as a control.

Fig. 6. Transgenic expression of lesion cell death (*LCD*)2 and *LCD4* in Columbia-0 (Col) and Wassileskija-2 (Ws). Phenotypes of the 3-week-old transgenic plants with the Col alleles of *LCD2* and *LCD4* in Ws (left panel) and Col (right panel). Plants representative of each category are shown and underneath are the number of lines exhibiting that phenotype. *LCD4* induced the cell death phenotype in both Ws (47 of 67 lines) and Col (27 of 37 lines) whereas *LCD2* did not induce cell death in Ws (total 35) or Col (total 39) transgenic lines.

notype in Col and Ws. Together with the observation that the *LCD4* genes from Col and Ws code for the same amino acid sequences, it suggests that the natural variation of *LCD4* in *bon1bon3* might have resulted from variation in its RNA expression.

DISCUSSION

There are more than 200 *R*-like genes in the *Arabidopsis* genome and a dozen of them have been shown to play key roles in plant defense responses to various pathogens. However, the function and the regulation of the majority of them are largely unknown. Here, we revealed activities of a group of TIR-NB-LRR or TIR-NB-type *R*-like genes through their natural variations. These so-named *LCD* can quantitatively induce defense responses and cell death in the absence of the *BON1* and *BON3* function. In Col, the double mutant is lethal due to the activation of these genes in addition to *SNC1*. The loss-of-function of each of the *LCD* is capable of reducing the defense and cell death phenotype of the *bon1bon3* mutant. In Ws, *bon1-2bon3-1* exhibits a wild-type phenotype due to either a loss of *LCD* functions or the loss of their regulation by the *BON* genes.

All of the natural modifiers of *bon1bon3*, including the *LCD* proteins and *SNC1*, are of the TIR-NB-LRR type of *R* proteins except for *LCD4*, which has TIR-NB but not LRR. The TIR-NB proteins have not been isolated as *R* genes previously. However, truncated forms of *R* proteins such as the CC-NB part of Rx and the TIR-NB part of RPP1A have been shown to lead to constitutive activation of defense responses (Moffett et al. 2002; Weaver et al. 2006). The discovery of *LCD4* indicates that naturally occurring TIR-NB-type proteins can be functional in cell death control.

The *LCD*, though all repressed in the wild type by *BON1* and *BON3*, may be regulated by the *BON* genes through different mechanisms. The *LCD* proteins fall into different subgroups of *R*-like proteins and the *LCD* genes do not appear to share common *cis*-acting regulatory sequences. In addition, their transcripts are differentially regulated by the *BON* genes and SA. One possible mode of *LCD* regulation could be at the protein activity level. Alterations in two amino acids in the LRR domain of the Ws *LCD2* might be sufficient to change its specificity so that it is not subject to the negative regulation by *BON1* and *BON3* as would be Col *LCD2*. Alternatively, these alterations can render the protein inactive, because structure-function studies of *R* proteins such as RPM1, RPS2, and N have found many missense mutations abolishing protein activities (Dinesh-Kumar et al. 2000; Tao et al. 2000; Tornero et al. 2002). Similarly, natural variations causing residue changes in the *R* protein TAO1 can lead to a loss of *R* function in a particular accession (Eitas et al. 2008). The second potential mode of regulation is at the transcript level due to differences in *cis*-regulatory elements or epigenetic controls. Polymorphisms between Col and Ws were found in the promoter sequences but not the protein sequences of *LCD4*. Introducing the Col *LCD4* into Ws as a transgene is sufficient to induce cell death, suggesting that *LCD4* might be regulated by *BON1* and *BON3* at the transcript level. Further studies should reveal how each *LCD* is regulated by the *BON1* family.

Evidently, these *R*-like genes have to be tightly controlled because their activation is detrimental to plant growth. Members of the *BON1* family apparently act as negative regulators of a significant number of such genes. The study here reveals at least four *LCD* as targets of *BON1* and *BON3*. Using VIGS to knock down *LCC* genes in *bon1bon3*, we found that *LCC9* is a potential *LCD*. The existence of opposite association of

QTL3 at the bottom of chromosome 1 indicates an additional target or targets of *BON1* and *BON3* and it is most likely an *R*-like gene as well. This *R*-like gene in Ws might induce a weak defense and cell death phenotype in *bon1-2bon3-1*, accounting for a slight upregulation of *PR1* in this mutant compared with the wild-type Ws. We expect that *BON3* has its own target gene or genes because *BON1* has its own target *SNC1*. *BON2*, the other member of the *BON1* family in *Arabidopsis*, likely has additional targets, either of its own or together with *BON1*. This is suggested by a lethal phenotype of the *bon1bon2* double mutants in both Col and Ws (H. Yang and J. Hua, unpublished data). The *BON1* and *BON2* targets could be *R*-like genes shared by Col and Ws or distinct *R*-like genes existing only in one accession but not the other. Adding all the *LCD*, *SNC1*, and the above-mentioned potential targets (two more of *BON1BON3*, one of *BON3*, one of *BON2*, one of *BON1BON2*, and one of *BON2BON3*) together, the *BON1* family could negatively regulate 11 or more *R*-like genes in *Arabidopsis*. This allows further mechanistic studies of regulation of *R*-like genes by the *BON1* family.

Given the large number of *R*-like genes regulated by *BON1* and *BON3* in Col, it seems remarkable that the Ws *bon1bon3* mutant looks like the wild type. At least two scenarios could be envisioned for the apparent lack of *R* genes activated in the Ws mutant. The effect of activation of some *R*-like genes is masked by other loci in Ws, which is supported by the finding of positive contributions from Ws at QTL3. It is also possible that the Ws accession has a lower temperature threshold for *R*-gene expression or activation. Some *R*-like genes might be activated in the Ws *bon1bon3* at temperatures <22°C. This accession difference might reflect an adaptation to the temperature environment at their original geographic locations. Consistent with this idea, the *bon1-2* mutant in Ws was observed to exhibit a reduced growth phenotype at 16 but not 22°C. It will be interesting to analyze the Ws *bon1bon3* mutant at lower temperatures.

This study also reveals the potentially quantitative nature of some *R*-like genes. *R* genes are usually responsible for qualitative disease resistance, with a major effect in disease resistance as identified in “gene-for-gene” interactions. Disease resistance can also be quantitative, and durable resistance is often determined by many minor genes or QTL. Genes with quantitative contribution to disease resistance are usually not associated with *R*-like genes. Recent studies, however, reveal that some *R* genes act additively to generate full disease resistance. For instance, the effector protein AvrB activates RPM1 and TAO1, and these two NB-LRR proteins then act additively to generate a full disease resistance response to *P. syringae* expressing this effector (Eitas et al. 2008). Similarly, the AvrRPM1 effector triggers defense mediated by both RPM1 and RPS2 (Kim et al. 2009). The identification of multiple *R*-like genes as QTL in *bon1bon3* suggests that *R*-like genes could act quantitatively as well. These *R*-like genes could function as major *R* genes for some particular pathogens and contribute quantitatively to other pathogens. Alternatively, these *R* genes are in the process of evolution, departing from the previous major *R* function but yet to acquire a new major *R* function. Various degrees of cell death phenotype in *bon1bon3* RIL result from activation of different combinations of *R*-like genes. The L5 plants showed a stronger cell death phenotype than their Col parent, possibly due to the presence of the Ws QTL3 where several clusters of *R* genes are located. The genetic interaction of the *BON* genes and multiple *R*-like genes creates a wide range of variation in cell death phenotype. These phenotypes are strongly influenced by environmental factors, especially temperature. Both *bon1* and *bon1bon3* mutants exhibit wild-type morphol-

ogy when grown at higher temperatures and, thus, the lethality or cell death can be masked under some environmental conditions.

The *BON1* family appears to act as master repressors of a number of *R*-like genes. This might be critical for the evolution, especially the expansion, of this type of gene in plants because improper activation of defense response is detrimental to plants. This intriguing genetic interaction together with the environmental modulation of the *bon* phenotypes could be a mechanism for creating hybrid barriers (Bombliès et al. 2007). As proposed in the Dobzhansky-Muller model, hybrid incompatibilities are caused by the interaction between genes that have functionally diverged in the respective hybridizing species. The *BON1* family could keep the newly acquired *R* genes in an inactive state, allowing them to evolve new functions and specificities for pathogens. Accumulation of *LCD* in the presence of *BON1* and *BON3* is tolerated by *Arabidopsis* but leads to lethality when *BON1* and *BON3* are not functional. The dependence of genetic background for the acquisition of new *R* genes is also recently revealed in hybrid necrosis. In *A. thaliana*, approximately 2% of intraspecific crosses among accessions yield F1 progeny with extensive necrosis under their natural growth conditions (Bombliès et al. 2007). In at least one case, an NB-LRR gene in one accession is necessary and sufficient to induce cell death when combined with a second gene in another accession. In sum, the essential function of the *BON1* family points to the necessity of negative regulation of *R*-like genes. Further study should reveal the molecular mechanism by which *R*-like genes are regulated by the evolutionarily conserved *BON* genes.

MATERIALS AND METHODS

Plant growth conditions.

Arabidopsis seed were directly planted on soil or sown on petri dishes containing half-strength Murashige-Skoog medium (Sigma-Aldrich, St. Louis) with 2% sucrose and 0.7% agar and suitable antibiotics. *Arabidopsis* plants were usually grown at 22 or 28°C under continuous fluorescent light (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and 50 to 70% relative humidity. Plants for the pathogen test were grown under a photoperiod of 12 and 12 h, day and night, respectively.

Genotyping.

PCR-based molecular markers were designed according to the available DNA sequencing data to differentiate the polymorphism between Col-0 and Ws-2.

RT-PCR.

For RT-PCR, total RNA was extracted from 3-week-old seedlings using Tri Reagent solution (Molecular Research Center, Cincinnati, OH, U.S.A.). RNA samples were treated with RNase-free DNase I (Promega Corp., Madison, WI, U.S.A.) and used as templates for the first-strand cDNA synthesis followed by PCR amplification with the ProtoscriptII RT-PCR kit (New England Biolabs, Beverly, MA, U.S.A.) following the manufacturer's instruction.

Plasmid construction and plant transformation.

BAC clone F11A6 from the ABRC was used to clone the *LCD2* and *LCD4* genomic fragments. An *EcoRV/XbaI* fragment containing *LCD2* and a *StuI/EcoRI* fragment containing *LCD4* were cloned into binary vector pCambia1300. Both include the promoter (at least 1.5 kb), coding and the 3' UTR regions (at least 1 kb). The constructs were transformed via *Agrobacterium tumefaciens* strain GV3101 into *Arabidopsis* plants by floral dipping method (Clough and Bent 1998).

Isolation and generation of single, double, and triple mutants.

All the *bon* and *snc1* mutants were described previously (Yang et al. 2006a). All the *lcc* mutants were obtained from the ABRC. F2 populations were analyzed by PCR-based genotyping to obtain the desired mutant combinations.

VIGS analysis.

A genomic fragment with <85% homology to other genes was chosen for VIGS. Each gene was amplified via PCR and cloned into the TRV2 vector as instructed (Burch-Smith et al. 2006). The *bon1-lbon3-3* plants were grown at 28°C for 2 weeks and VIGS was performed as described (Burch-Smith et al. 2006). Plants were transferred to 22°C 3 days after infiltration and phenotypes were analyzed 2 weeks after the transfer.

Histochemical staining and pathogen resistance assay.

DAB staining was carried out as described (Yang et al. 2006a). Pathogen resistance assay was carried out as described (Li et al. 2007).

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 CAMBIA Initiative for Open Innovation pCAMBIA vector webpage:
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 Center for Biological Sequence Analysis (CBS) Prediction NetGene 2
 server: genome.cbs.dtu.dk/services/NetGene2
 Multiple SNP Query Tool:
msqt.weigelworld.org/cgi-bin/nordborg/msqt-sbe.cgi
 North Carolina University's Statistical Genetics and Bioinformatics
 website: statgen.ncsu.edu/qlcart/Zmapqtl.php
 Sanger Institute Pfam database: pfam.sanger.ac.uk
 TRES Transcription Regulatory Element Search program:
bioportal.bic.nus.edu.sg/tres