Enhancement of Rice Resistance to Bacterial Blight by Overexpressing *BrCP3* Gene of *Brassica rapa*

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ABSTRACT *Brassica rapa* cysteine protease 3 (*BrCP3*) is an endopeptidase member of C1A family (papain-like) and CA clan of cysteine protease. Members of papain-like cysteine protease (CP) have emerged to be key enzymes involved in cell death in response to biotic and environmental stresses. Recent progress of CP research features its diverse and overlapping role in basal and effector-triggered immunity. This study was carried out to characterize *BrCP3* gene in Chinese cabbage and explore its function in rice – *Xanthomonas oryzae* pv. *oryzae* pathosystem. Phylogenetic analysis and BLASTP search of its amino acid sequence to the Arabidopsis Protein TAIR 10 database identified *BrCP3* as the closest ortholog of Arabidopsis RD21a. The full-length cDNA of *BrCP3* was cloned and transformed into the genome of japonica rice cv. ‘Gopum’. Constitutive overexpression of the transgene is driven by Ubi-1 promoter in *pFLCIII* vector. Regenerated single copy overexpression rice lines were inoculated with three *Xoo* Korean isolates. Regulation of rice resistance through cysteine protease activity is evident in the overexpression lines which showed enhanced immunity to three isolates as depicted by reduced lesion length compared with the wild type. Our initial results implicate an interesting biological function of *BrCP3* in rice system that has never been reported before.

Keywords *Brassica rapa*, Cysteine protease 3, BrCP3, Bacterial blight, Rice, *Xanthomonas oryzae* pv. *oryzae*

INTRODUCTION

Plants are known to possess sophisticated mechanisms to perceive pathogen attack and activate innate immune response. A number of studies demonstrate that plants have evolved multiple defense strategies for combating invading pathogens. In some cases, plants utilize proteolytic machinery to halt pathogen invasion. Among them is cysteine protease which activity comprises majority of the proteolytic activities in plants. It shares approximately 30% of total proteolysis in mature non-senescing organs, and in some cases rises to 90% in response to stimuli (Grudkowska and Zagdanska 2004). MEROPS database showed that papain-like cysteine proteases (PLCPs) are the most abundant in nature being

present in numerous species of bacteria, archaea, protozoa, fungi, plants, animals, and viruses (Rawlings *et al.* 2014). Most members of this clan are pre-proteases containing a signal sequence, an autoinhibitory prodomain, and a mature protease domain (van der Hoorn *et al.* 2004; Niño *et al.* 2014). The activity of many proteases also depends on pH, indicative of the compartment where they localize, and on the presence of endogenous protease inhibitors or activators (Beynon and Bond 2000). CA clan is further sub-grouped into families. C1 family is subdivided into C1A, an extracytoplasmic PLCPs that possess disulfide bridges and accumulate in the vesicles, vacuole, and apoplast, and the subfamily C1B which comprises cytoplasmic PLCPs (subfamily C1B) (Rawlings *et al.* 2006; Shindo and van der Hoorn 2008). A total of 169 plant

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species were recorded in the MEROPS database to contain CP assigned to this family (Rawlings *et al.* 2014). Of which, 11 species were shown to contain more than 30 homologues in their genome including *Glycine max*, rice, *Arabidopsis lyrata* and *Zea mays*, among others (Niño *et al.* 2014). But despite the abundance of cysteine proteases in plants, only a few were reported. In *Arabidopsis* alone, functions for only a few of the more than 550 peptidases (Rawlings *et al.* 2014) have been studied genetically (Adam and Clarke 2002; Beers *et al.* 2004; van der Hoorn *et al.* 2004).

Reports have been made on papain-like cysteine proteases acting as inhibitory agent against fungus, bacteria, oomycete and nematode such as *Rcr3* (Krüger *et al.* 2002), *PIPI* (Tian *et al.* 2007), *RD19* (Bernoux *et al.* 2008), *RD21* (Shindo *et al.* 2012), *C14* (Kaschani *et al.* 2010) and *Cath B* (Gilroy *et al.* 2007). Each has unique participation in the induction of host immunity which is evident in the specific interactions with the effectors, involvement in a specific molecular battlefield, and the resulting immunity threshold (Niño *et al.* 2014).

In Korea, bacterial blight is an emerging disease causing damage to rice yield and grain quality (Noh *et al.* 2007). *Xanthomonas oryzae* pv. *oryzae* (Xoo), a gram negative bacterium, is the causal pathogen of bacterial leaf blight. It is the most serious bacterial disease of rice in many rice-growing areas around the globe. It invades the host plant either through wounds or hydathodes, multiplies in the epitheme and moves to the xylem vessels where active multiplication occurs (Niño-Liu *et al.* 2006). What made this pathogen difficult to control is their ability to evolve swiftly as indicated by emergence of various pathotypes and races. Studies using molecular genotyping methods on diversity, distribution of *X. oryzae* pv. *oryzae*, and the relationship between phylogeny and virulence have provided better information on population structures and their evolution and have indicated that BB pathogen populations from different Asian countries were distinct (Leach *et al.* 1992; Nelson *et al.* 1994; Adhikari *et al.* 1995). The population structure of Korean Xoo races is markedly distinct from Philippines (64% distance) and Japan (58% distance). Recent pathotyping results also suggested that Korean isolates had continuous genetic

variations; therefore, they might have had unique hierarchical evolutionary pathways for their pathogenicity (Jeung *et al.* 2006). Korean race K1 showed a decreasing trend by the spread of rice cultivars with *Xa1* and *Xa3* genes. However, races K2 and K3 have increased in Korea. The new pathotype, K3a that had reaction scores identical to those of race K3 against Korean differential lines caused severe BB epidemics in 2003 (Noh *et al.* 2003).

This study was conducted to characterize the cysteine protease (*BrCP3*) in Chinese cabbage, and to clone and overexpress its full-length cDNA into japonica rice to investigate if it plays any role for resistance to Korean Xoo isolates. Our initial findings reveal interesting biological function of the gene in rice – Xoo pathosystem.

MATERIALS AND METHODS

Isolation of *BrCP3* gene

The total RNA was extracted from the young leaves of *Brassica rapa* cv. Osome using the Qiagen kit (Qiagen, Korea, Ltd.). About 1 µg of high quality RNA was utilized to construct the cDNA library using oligo (dT) primer (Invitrogen, Carlsbad, CA, USA). The cDNA was then used as a template for PCR amplification using *BrCP3*-specific primer pair, 5' tagtgaagaaaaatgaagctcc 3' (forward) and 5' gtgtcaccaacttcttacgc 3' (reverse), which were designed from the reference gene LOC103848250. The target gene was amplified using the following condition: denaturation at 95°C for 5 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 2 min, and a final extension at 72°C for 10 min. The PCR product was electrophoresed on 1% (w/v) agarose gel stained with ethidium bromide. The expected amplicon with the right band size was eluted using the gel purification kit (Bioneer, CA, USA). The purified PCR product was sub-cloned into pGEMT-easy vector (Promega, Madison, USA) and sent to Macrogen Inc. (Macrogen, Seoul, Korea) for sequence analysis. Sequence homology was verified using multiple sequence alignment with hierarchical clustering.

Vector construction and transformation

The full-length cDNA of *BrCP3* was ligated at the two

Fig. 1. Schematic diagram of the binary Ti plasmid pFLCIII containing the full-length cDNA of *Brassica rapa* cysteine protease 3. The vector consists of two different *SfiI* sites [*SfiI*(A) and *SfiI*(B)]. P35S, CaMV 35S promoter; pUbi-1, maize Ubiquitin-1 promoter; Tg7 and Tnos, polyadenylation signals from gene 7 and nopaline synthase (nos) gene in the T-DNA; hygromycin resistance gene (HPT); LB, left border; RB, right border.

sfiI sites (*sfiI* A and *sfiI* B) of *pBig_sfiI* vector (Takara, Shiga, Japan) (Fig. 1) using the T4 ligase kit (Promega, Madison, USA). Overexpression of this insert gene was constructed under the control of ubiquitin-1 promoter. The binary vector also carries *hygromycin phosphotransferase* (HPT) gene as a selectable marker (Abdula *et al.* 2013). The ligated product was transformed into *Agrobacterium tumefaciens* strain EHA105, which was then cultured on AB medium containing 50 mg/L kanamycin sulfate solidified with 1.5% microagar for three days at 28°C in the dark. *Agrobacterium* culture was harvested and suspended in AAM medium to yield an OD₆₀₀ of 0.01. Ten-day-old cultured rice seed calli were immersed in the *Agrobacterium* suspension by gently inverting the tube for 3 min and blotted dry. The inoculated calli were placed on 2N6-AS medium solidified with 0.4% gelrite (Lee *et al.* 2011).

After three days of co-cultivation at 25°C in the dark, calli were washed with sterile distilled water treated with 500 mg/L carbenicillin. The calli were blotted dry and planted on 2N6 medium treated with 50 mg/L hygromycin and 400 mg/L carbenicillin under continuous light at 32°C for two weeks. Proliferating calli were transferred to MSR medium for shoot and root induction. Regenerated plants were transplanted to soil (a mixture of 50% composed peat and 50% earth soil) in the greenhouse and acclimatized for two weeks.

DNA extraction and genomic PCR

Genomic DNA was extracted as described in Cho *et al.* (2007) with some modifications. The relative purity and concentration of extracted DNA was estimated using NanoDrop-1000 (NanoDrop Technologies). To verify successful integration of *BrCP3* and *hygromycin*

phosphotransferase (HPT) gene, PCR assay was performed using the Takara ExTaq (Takara, Shiga, Japan) and internal primer sets: 5'GGTCGGATACGGAACAGAGA 3' (forward), 5'GGAGGCGTAACGGGAGAT 3' (reverse) for *BrCP3* and 5'GGATTTCGGCTCCAATGTCCTGACGGA3' (forward), 5'CTTCTACACAGCCATCGGTCCAGA3' (reverse) for HPT. The target genes were amplified using the following condition: denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, and a final extension at 72°C for 5 min. The PCR product was electrophoresed on 1% (w/v) agarose gel stained with ethidium bromide and viewed under UV light.

TaqMan real-time PCR

To detect and measure transgene copy number variation within the transgenic rice, TaqMan[®] copy number assay was performed using the TaqMan[®] Reagents (Applied Biosystems, CA USA). The whole process was done based on the TaqMan[®] copy number assay protocol. In a copy number quantitation reaction, purified genomic DNA obtained from T₀ plants was combined with: TaqMan[®] Copy Number Assay which contains two primers and a FAM[™] dye-labeled MGB probe to detect the genomic DNA target sequence; TaqMan[®] Copy Number Reference Assay which contains two primers and a VIC[®] dye-labeled TAMRA[™] probe to detect the genomic DNA reference sequence; and TaqMan Genotyping Master Mix which contains AmpliTaq Gold[®] DNA polymerase, ultrapure, and dNTPs. The real-time PCR reaction condition was set as follows: enzyme activation at 95°C for 10 min, denaturation at 95°C for 15 sec, and annealing at 60°C for 60 sec. After amplification, data files were analyzed using Applied Biosystems CopyCaller[™] software for copy

number quantitation. The number of copies of the target sequence in each test sample is determined by relative quantitation (RQ) using the comparative C_T ($\Delta\Delta C_T$) method. This method measures the C_T difference (ΔC_T) between target and reference sequences, then compares the ΔC_T values of test samples to a calibrator sample(s) known to have two copies of the target sequence. The copy number is calculated to be two times the relative quantity.

Gene expression analysis

To characterize the expression of *BrCP3* gene, total RNA were extracted from the different tissues of *B. rapa* cv. Osome using the RNeasy Mini Kit (Qiagen). Total RNA were cleaned using DNase 1 kit (Invitrogen), and the first-strand cDNA was synthesized using oligo(dT)₂₀ primers with SuperScriptTM III Reverse Transcriptase (Invitrogen). *BrCP3*-specific internal primers were used for quantitative RT-PCR. Actin primers were used as loading controls and also as internal controls to normalize the results of the quantitative RT-PCR reaction.

Bacterial blight screening

The T₂ transgenic plants were pre-germinated on trays and transplanted in the pots. Treatment controls were provided including the wild type ‘Gopum’, susceptible check ‘IR24’, and resistant check ‘IRBB21’. Seedlings were allowed to grow for 45 days in the greenhouse prior to inoculation. Three replications were provided for each treatment with three plants per replication. Bacterial inoculum was prepared from a 48 hr-old culture of *Xanthomonas oryzae* pv. *oryzae* (Korean isolates K1, K3, K3a) grown on a plated peptone sucrose agar medium. The culture plates were harvested, diluted with sterile distilled water, and the concentration was adjusted to 1×10^8 cfu/ml. The plants were inoculated by leaf-clipping method and incubated for 14 days. Scoring was done by measuring the lesion length in cm. The average value was interpreted using the standard evaluation system (SES IRRI 1996).

Bacterial growth assay

Using the same set up mentioned above, sampling was done three days after inoculation. Since K3a is the newest race to develop among the three *Xoo* races in Korea, only

samples from this treatment were used for the assay. About 0.5 cm² leaf tissue was collected from each treatment plant and immediately washed with 70% ethanol and 1% sodium hypochlorite followed by three changes of sterile distilled water. The tissue was macerated aseptically in a 1.5 ml tube containing 100 µl sterile distilled water. A serial dilution of 10², 10⁴, 10⁶ and 10⁸ were plated onto peptone sucrose agar medium. The plate was sealed and incubated at 30°C for two days. Number of colonies was counted and the number of bacteria (cfu) per milliliter of sample was calculated by dividing the number of colonies by the dilution factor, multiplied by the amount of specimen added to liquified agar.

Field performance evaluation

The four T₃ transgenic lines and the wild type were sown in planting trays and kept in greenhouse for three weeks. The seedlings were transplanted in the experimental farm at 30×15 cm spacing with one seedling per hill. The experiment was laid out in randomized complete block design (RCBD) with five replications. Each transgenic rice line was planted in 2×2 m² plot size. The fertilizer N-P₂O₅-K₂O were applied at the rate of 90-45-47 kg/ha. The morpho-agronomic characters were evaluated at appropriate growth stages of the transgenic rice (Sun *et al.* 2011).

Analysis of variance (ANOVA) was computed using the Statistix version 10. Significant *P* value was further analyzed using the two-sided Dunnett’s multiple comparisons with the wild type as control.

Web-based tool analysis

The target gene sequence was verified using the BLAST program in NCBI database. Conserved domain was generated by NCBI BLASTN program (<http://www.ncbi.nlm.nih.gov/>). Sequence alignment and ORF translation were performed using DNASTar’s Lasergen sequence (www.dnastar.com).

RESULTS

Characterization of *BrCP3* gene

Analysis of *Brassica rapa* cysteine protease 3 (*BrCP3*)

sequence revealed that it has a full-length cDNA of 1,374 bp with 60 bp-long of 5' and 170 bp of 3'UTR which encodes for 457 amino acids (Fig. 2A). Its conserved domain has high affinity to peptidase_C1A subfamily of the clan CA of cysteine protease (Fig. 2B). It contains Cys and His forming a catalytic dyad as catalytic residues. Two other residues play an important role in catalysis: a Gln preceding the catalytic Cys, believed to help in the formation of the oxyanion hole; and an Asn residue which orients the imidazolium ring of the catalytic His (Rawlings and Barrett 1993). It is an endopeptidase containing a signal sequence, a cathepsin propeptide inhibitor domain (129) in the N terminus, and a granulin domain in the C terminus. Members of CA contain proteases with a papain-like fold (van der Hoorn 2008). Papain has specific substrate preferences, primarily for bulky hydrophobic pocket or aromatic residues at the S2 subsite, a hydrophobic pocket in papain that accommodates the P2 sidechain of the substrate.

Phylogenetic analysis using deduced amino acid sequences of reported papain-like cysteine proteases in plants showed that *BrCP3* is most closely related to Arabidopsis cysteine protease, *RD21A* (At1g47128) with 82.49% homology (Fig. 2C). Further alignment analysis of their conserved domains showed that both are 83.93% similar in prodomain, 89.25% in protease domain, and 96.49% in granulin domain (data not shown).

RD21A is highly overexpressed in the leaf of Arabidopsis particularly during senescence indicating its role in cell death (Shindo *et al.* 2012). Similarly, *BrCP3* gene is also remarkably overexpressed in the leaf tissues, about 25-fold higher than in roots and stems of *Brassica rapa* although the highest levels, 2.48- and 1.39-fold higher than the leaves, were observed respectively in sepal and stamen (Fig. 3A). Expression in the petal, flower bud, and pistil is also higher compared to roots and stem tissues. Temporal expression analysis also revealed that *BrCP3* is highly

Fig. 2. *BrCP3* sequence analysis. (A) Nucleotide and deduced amino acid. (B) Conserved domain structure of *BrCP3*. (C) Phylogenetic analysis of commonly reported papain-like cysteine protease based on amino acid sequences.

Fig. 3. Transcriptional expression pattern of *BrCP3* in the different tissues (A) and flower bud stages (B) of *Brassica rapa* cv. Osome.

activated at 1 and 2 week-flower bud stages (Fig. 3B). Levels start to decrease at 3 which sustained until 6 weeks.

Development of *BrCP3*-overexpressing transgenic rice

The *pBig_sfiI* vector was constructed with the full-length cDNA of *BrCP3* along with the *HPT* gene marker. To facilitate overexpression, integration of the transgenes was constructed under the control of CaMV 35S and ubiquitin-1 promoter and NOS terminator (Abdula *et al.* 2013). The whole construct was transformed into japonica rice cv. ‘Gopum’ using *Agrobacterium*-mediated transformation method, and regeneration of transgenic plants was carried out through tissue culture (Lee *et al.* 2011). The T_0 plants were evaluated for gene copy number using TaqMan copy number assay, a duplex real-time polymerase chain reaction (PCR) which run TaqMan Copy Number Assays simultaneously with a TaqMan Copy Number Reference Assay. The Copy Number Assay detects the target gene and the reference assay detects the positive control sequence that is known to exist in two copies. Of the ten T_0 plants assayed, four were confirmed to carry single copy of the gene (Fig. 4). These include BCP4 (1.37), BCP6 (0.92), BCP9 (0.96), and BCP10 (1.28). Five plants (BCP1,-2,-3,-7,-8) were found to carry two copies while BCP5 carries 3.

Fig. 4. Copy number of T_0 *BrCP3* overexpression rice plants assayed using TaqMan real-time PCR.

These single gene copy plants were forwarded to subsequent generations for further analyses. Integration of the transgenes *BrCP3* and *HPT* was verified in the subsequent generations through genomic PCR (Fig. 5A). Since the transgene was overexpressed under the control of CaMV 35S and ubiquitin-1 promoter, it is important to

Fig. 5. Molecular characterization of overexpression lines. (A) Genomic PCR assay for the presence of *BrCP3* and HPT. (B) Expression of *BrCP3* and actin in the different tissues of transgenic plants.

Fig. 6. Evaluation of overexpression lines for resistance to *Xoo*. (A) Lesion length of bacterial blight infection 14 days after inoculation in the leaves of *BrCP3* transgenic plants, wild type Gopum, susceptible check IR24 and resistant check IRBB21. Three *Xoo* isolates (K1, K3 and K3a) were used for infection at 1×10^8 cfu/ml. (B) Bacterial concentration in 0.5 cm^2 leaf tissue at 10^6 dilution factor three days after inoculation.

verify if indeed the gene was constitutively overexpressed in the whole parts of the recipient cultivar. RT-PCR assay revealed that *BrCP3* is transcriptionally overexpressed in the whole young seedling, leaf, flower, stem, and root tissues of transgenic rice (Fig. 5B).

BrCP3* improves resistance to *Xoo

The *BrCP3* transgenic plants showed enhanced resistance to *Xoo* (Fig. 6A). Lines inoculated with K1 showed lesion length that ranged from 1.8 to 2.6 cm, which are about half shorter than the wild type (4.1). Surprisingly, resistant

check (IRBB21) also displayed sensitive response similar to the susceptible check (IR24). For K3 treatment, transgenic lines displayed lesions ranging from 0.95 to 3.10 cm which are shorter than the wild type (6.52) and IR24 (11.62) and relatively similar with the IRBB21 (2.43). For K3a, the transgenic lines had lesions that ranged from 1.2 to 2.8 cm, which are shorter than the wild type (6.6), IRBB21 (5.1) and IR24 (32.8). SES index rating indicates that response of all overexpression lines were categorized as highly resistant (HR) to K1, while they are highly to moderately resistant (MR) both to K3 and K3a. The wild type Gopum which displayed a much longer infection is MR to K1 and moderately susceptible (MS) both to K3 and K3a.

Quantification of leaf bacteria (K3a) revealed that *BrCP3* indeed showed improved resistance (Fig. 6B). Among the dilution factor, number of colonies at 10^6 is the one showing the best count. Colonies at the dilution factor of 10^2 and 10^4 are too many to count while colonies at 10^8 are too few to count. The concentration of bacteria was 0.97×10^8 in *BrCP3* transgenic which is relatively lower than 2.31×10^8 in Gopum, 1.78×10^8 in IRBB21 and 9.05×10^8 in IR24. Data on *in vitro* and *in vivo* screening against *Xoo* complemented well supporting our finding that overexpression of *BrCP3* confers resistance to *Xoo*.

Characterization of transgenic plants

The four transgenic lines (BCP4, BCP6, BCP9, BCP10) were evaluated for agronomic characters in the field (Table 1). Data showed that these lines are not significantly different

with the wild type Gopum in terms of number of productive tiller, culm length, and panicle length. One line (BCP9), however, is significantly shorter (with 7 cm difference) than the wild type which is more likely contributed by shorter panicle length displayed by this line.

DISCUSSION

Brassica rapa cysteine protease 3 (*BrCP3*) belongs to the family C1A (papain-like) and clan CA in the Merops protease database. As member of papain-like CP, this gene is synthesized as inactive proenzyme with N-terminal propeptide regions, which are removed upon activation. Residues within its propeptide region play a role in the transport of the proenzyme to lysosomes or acidified vesicles. Sequence homology analysis of its deduced amino acid against selected known plant cysteine proteases revealed its close relationship with *Responsive-to-Dessication-21* (RD21A) in Arabidopsis. BLASTP search of its amino acid sequence to the Arabidopsis Protein TAIR 10 database verified that *BrCP3* is the ortholog of Arabidopsis RD21a (E-value 0.0). Further phylogenetic analysis of the amino acid sequences of the conserved domain showed a very high similarity in prodomain (83.93%), protease domain (89.25%), and granulin domain (96.49%). RD21A is expressed in the key tissues of Arabidopsis including leaf, flower, sepal, stamen, stem, among others. Remarkably, it is highly expressed in the leaf undergoing senescence implying its role in cell death. Similarly, *BrCP3* is also

Table 1. Morphological characteristics of the T₃ transgenic lines established in the field.

Lines	Productive Tiller	Plant Height (cm)	Culm Length (cm)	Panicle Length (cm)
BCP4	13.0±0.83	110.2±11.72	77.0±10.58	21.2±6.23
BCP5	11.4±0.98	110.5±6.23	77.0±8.01	20.9±2.29
BCP9	12.4±0.59	106.2±8.30*	76.9±7.79	20.3±6.77
BCP10	11.2±1.14	109.8±10.59	77.6±6.32	21.2±1.88
Gopum	11.8±0.70	110.4±14.44	77.2±12.27	21.0±9.63
DMC ₀₅	0.15	4.79	1.98	0.03
CV(%)	5.69	1.01	1.14	3.33

DMC=Dunnnett's multiple comparison were used to determine the significance (5% level) of *BrCP3* plants with the wild type Gopum. Asterisk (*) means significant from the wild type at 5% level. CV=coefficient of variation. Data presented are mean±SE.

highly expressed in sepal, stamen, leaf and flower bud.

The full-length cDNA of *BrCP3*, which is 1,374 bp-long, was isolated from the *Brassica rapa* cv. Osome and integrated into the *pBig_sfiI* binary vector along with HPT marker. Overexpression of these genes is driven by CaMV 35S and ubiquitin-1 promoter to facilitate constitutive expression. The vector construct was transformed into the genome of japonica rice cv. Gopum. *Brassica rapa* is distantly related to *Oryza sativa* hence introduction of *BrCP3* has low chances of effecting gene silencing, a phenomenon well-observed in endogenous genes/transgenes in eukaryotes (Kishimoto *et al.* 2013), which happens when the genes involved share sequence homology in their promoter regions (Matzke *et al.* 2002; Wassenegger 2002). Four transgenic rice plants overexpressing a single copy of the gene were developed and used for the analyses in this study. Field performance test showed no aberrant morphological characters observed in three transgenic lines (BCP4, BCP5, BCP10) in comparison with the wild type indicating no major genomic changes occurred in the transgenic rice. Induced infection assay of three *Xanthomonas oryzae* pv. *oryzae* (Xoo) races (K1, K3, K3a) revealed enhanced immunity to the disease. Although the wild type displays some degree of resistance, where it is moderately resistant to K1 and moderately susceptible to both K3 and K3a, lesions were remarkably reduced in all overexpressing lines. The transgenic were highly resistant to K1 and highly to moderately resistant against K3 and K3a. To date, at least five races of *Xoo* have been reported in Korea. The new pathotype K3a was reported to cause severe BB epidemics in 2003 (Noh *et al.* 2003). Results of our screening test implicate that resistance conferred by cysteine protease to three *Xoo* isolates can be employed to regenerate damaged immunity in the host.

Cysteine proteases have emerged as key enzyme involved in the regulation of programmed cell death (PCD) both in animals and plants (Solomon *et al.* 1999). In plants, PCD has been implicated in xylogenesis (Fukuda 1996; Groove *et al.* 1997), in senescence, and in the hypersensitive response to pathogens and abiotic stresses (Greenberg 1996; Mitler and Lam 1996; Lamb and Dixon 1997; Solomon *et al.* 1999). Recent progress of cysteine protease research in plant disease immunity highlights its diverse

and overlapping role in basal and effector-triggered immunity. PLCPs are important target of pathogen effector guarded by other R genes for defense (Lozano-Torres *et al.* 2012). Nucleocytoplasmic trafficking of a few members such as RD19 implies that PCLPs are involved in the crucial transcriptional regulation of defense-related genes in the host (Bernoux *et al.* 2008). RD21A, the CP ortholog of *BrCP3* in *Arabidopsis*, exhibits immunity specifically against a necrotrophic fungal pathogen *Botrytis cinerea* but failed to fend off obligate biotrophic oomycete and *Pseudomonas syringae* (Shindo *et al.* 2008). The case, however, is different from our study where *BrCP3* confers resistance to an obligate biotrophic pathogen *Xoo*. *BrCP3* is just one of the several CPs in Chinese cabbage but to our knowledge, this is the first study conducted exploring its potential function to disease immunity. A detailed understanding on how CP halts *Xoo* remains to be seen. Nevertheless our initial findings set clues on the probable role of *BrCP3* in the nodes of immunity (perception, signaling, and execution) under rice – *Xoo* pathosystem.

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