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Original Paper

Enhanced resistance to gray mold (*Botrytis cinerea*) in transgenic potato plants expressing thionin genes isolated from Brassicaceae species

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Abstract Five novel thionin genes have been isolated from three Brassicaceae species, *Brassica oleracea* var. *acephala*, *Nasturtium officinale*, and *Barbarea vulgaris*. Comparison of the deduced amino acid sequences shows that these thionin proteins share seven highly conserved cysteine residues. These five thionin genes were, respectively, inserted between cauliflower mosaic virus 35S promoter and nopaline synthase terminator in a binary vector pEKH2. Transgenic potato plants were generated through *Agrobacterium*-mediated transformation methods. Southern blot analysis of transgenic potato plants indicated successful integration with varying copy number of the thionin genes into the plant genomes. Expressions of thionin transgenes in the transgenic plants were confirmed by RT-PCR and their protein products were immunologically detected by western blot analyses. Antifungal assay using detached leaves from transgenic lines expressing either of the thionin genes showed similar levels of enhanced resistance to *Botrytis cinerea* as compared to those of non-transformed control plants. Novel thionin genes isolated in this study are probably useful tools to produce transgenic plants resistant to various phytopathogens.

Key words: Antifungal resistance, Brassicaceae species, pathogenesis-related (PR) protein, *Solanum tuberosum*, thionin.

Potato (*Solanum tuberosum* L.) is one of the most important non-cereal food crops in the world. Potato production has been threatened by several fungal diseases and other microbial pathogens, resulting in 20% yield loss (Baker et al. 1997; James et al. 1990; Walter et al. 2011). Although *Botrytis cinerea* is not a major disease in potato, it is a good model system for other serious diseases such as *Fusarium solani* and *Phytophthora infestans*. Recently, transgenic approaches using genes encoding pathogenesis-related (PR) proteins have been shown to confer resistance to fungal pathogens (Broekaert et al. 1995, 1997; Cammue et al. 1992; Gao et al. 2000).

PR proteins, which have been found in many plant species to date, are classified into 17 families based on molecular properties of their antimicrobial activities against fungal or bacterial pathogens. Glycolytic enzymes, such as chitinase and glucanase, are PR proteins that digest components in cell wall of pathogens (Schroder et al. 1992). Anand et al. (2003) reported that wheat plants co-expressing a chitinase and β-1,3-glucanase genes obtained resistance to *Fusarium graminearum*. Also, transgenic potato plants expressing the alfalfa antifungal peptide (alfAFP) showed strong defense activity against fungal pathogen, *Verticillium dahliae*, under greenhouse and field conditions (Gao et al. 2000).

Thionins, another family of the PR proteins, are low-molecular-weight (5 kDa) cysteine-rich proteins with anti-microbial activity. There are many kinds of thionins, most of which mainly accumulate in seeds of higher plants. But some thionins exist in stems, roots, or leaves in tissue specific manner (Bohlmann 1994; Bohlmann et al. 1998). Thionins are toxic to a broad spectrum of phytopathogens, presumably attacking the cell membrane to increase their permeability. The increased permeability causes the death of fungal cells because of leakage of proteins, nucleotides, and other components (Cammue et al. 1992; Chan et al. 2005; Epple et al. 1997; Holtorf et al. 1998; Terras et al. 1995). Kanzaki et al. (2002) reported that transgenic rice over-expressing gamma-thionin gene from wasabi (*Eutrema wasabi*), enhanced resistance to rice blast disease caused by *Magnaporthe grisea*. In addition, Almasia et al. (2008)

Abbreviations: AFP, antifungal peptide; Bo, *Brassica oleracea*; Bv, *Barbarea vulgaris*; CTAB, cetyl-trimethyl ammonium bromide; DIG, Digoxygenine; GUS, β-glucuronidase; IAA, indole-3-acetic acid; MS, Murashige and Skoog; No, *Nasturtium officinale*; *npt*II, neomycin phosphotransferase II; PCR; Polymerase Chain Reaction; PDA, potato-dextrose agar; PR, pathogenesis-related; PVDF, polyvinylidine difluoride; RT-PCR, Rverse transcription-Polymerase Chain Reaction; SDS, sodium dodecyl sulphate.

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reported that transgenic potato plants over-expressing potato thionin (*snakin-1*) gene, enhanced resistance to *Rhizoctonia solani* and *Erwinia carotovora*. Also, transgenic potato plants, that over-expressed the wasabi thionin gene, had antifungal activity against gray mold (*Botrytis cinerea*) (Khan et al. 2006).

Several thionin genes have been identified in *Arabidopsis*, *Eutrema*, and *Raphanus* of the family Brassicaceae. N- and C-terminal amino acid sequences of these thionins are highly conservative but their nucleotide sequences differentiate to each other. Thus, a pair of degenerated primers was used to isolate five novel thionin genes from the Brassicaceae species. Each thionin gene was integrated into a binary vector and transgenic potato plants were produced by *Agrobacterium*-mediated transformation. Furthermore, antifungal resistance of the transgenic potato plants was evaluated by the infection with *Botrytis cinerea*. Transgenic plants expressing any thionin transgene showed increased level of resistance to *B. cinerea*, as compared to those of the non-transformed plants.

Three Brassicaceae species, *Brassica oleracea* var. *acephala* Giant kale, *Nasturtium officinale,* and *Barbarea vulgaris* variegated strain, were used to isolate thionin genes. Plant materials were grown in greenhouse and their leaves were collected. Leaf samples (*ca*. 100 mg) were frozen in 2-ml plastic tubes with liquid nitrogen and crushed into fine powder using a Multi-Beads Shocker (Yasui Kikai Co., Kyoto, Japan). Total genomic DNA was extracted according to the cetyl-trimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). A pair of degenerated primers, Thio5P: 5′-CGGATCCATGGCI(A/C)TT(T/C)GCITCIAT-3′ and Thio3P: 5′-CGTCGAC(A/G)CAIGG(A/G)AA(A/G)TA(A/G)CA(A/G/T)AT(A/G)CA-3′, were used for PCR amplification of DNA fragment containing coding sequence in the thionin gene. These primers were designed based on comparison among amino acid sequences of defensin-like protein (Genbank accession No. AY133787) in *Arabidopsis thaliana*, gamma thionin (AB012871) in *Eutrema wasabi*, and antifungal protein 1 (RSU18557) in *Raphanus sativus*. The restriction enzyme, *Bam*HI and *Sal*I (underlines), was introduced at the start and stop codon of Thio5P and Thio3P primer, respectively. PCR amplifications were performed as follows: 40 cycles of 94°C for 1 min (denaturation), 54°C for 1 min (annealing) and 72°C for 1 min (elongation) in a PTC200 thermocycler (MJ Research Inc., MA, USA). The amplified PCR products were subjected to 1.5% agarose gel electrophoresis and purified using a PCR purification kit (QIAquick, Qiagen Inc., CA, USA). The purified PCR products were cloned into pCR2.1 using TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and the resulting ligation products were transferred into *Eschrichia coli* TOP10 cells. The sequences of clones were determined using an automated DNA sequencer (ABI310; Applied Biosystems, CA, USA) with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). The sequences were analyzed using the NCBI web-based blast server (Altschul et al. 1990) and Genetyx Software ver. 15.0.5 (Software Development Co., Tokyo, Japan).

As listed in Table 1, five thionin genes, two (*BoT5*, *BoT6)* from *Brassica oleracea,* one (*NoT2*) from *Nasturtium officinale,* and two (*BvT2*, *BvT4*) from *Barbarea vulgaris,* that contained a single intron (98–107 bp) between first exon (40–64 bp) and second exon (176–194 bp), were identified and deposited to the DDBJ databank as accession Nos. AB683152–AB683156. All of the thionin genes isolated from the three Brassicaceae species contained a single intron. It was assumed to enhance expression of the reporter gene in transgenic plants (Ohta et al. 1990). Thus, the stop codon of the other four thionin genes except in *BvT4* which had an original stop codon, were removed to construct a fusion gene with *GUS* reporter gene. However, GUS activity could not be detected in transgenic plants (data not shown). We found that thionin protein has been reported to inhibit enzymatic activity of the fused *β*-glucuronidase (GUS) (Diaz et al. 1992). Therefore, the reconstructed binary vector eliminated *GUS* reporter gene and was used for the transformation experiments (Figure 1). Translation of the four thionin genes used in this study probably terminated at anonymous stop codon within the sequence of *nos* terminator because *GUS* gene was removed from each construct.

The amino acid sequences were aligned using ClustalW (Thompson et al. 1994). Although two stretches of 7 amino acid sequences, MAKVASI and CICYFPC, were completely conserved in N- and C-termini among eight thionin proteins, respectively,

Table 1. List of thionin genes isolated from three Brassicaceae species

Name	Species	1st exon	intron	2nd exon	ACC. NO
BoT5	*Brassica oleracea*	40	107	176	AB683153
BoT6	*Brassica oleracea*	64	98	176	AB683154
NoT2	*Nasturtium officinale*	64	99	176	AB683152
BvT2	*Barbarea vulgaris*	64	99	176	AB683155
BvT4	*Barbarea vulgaris*	64	100	194	AB683156

Base pair (bp) from start codon of 1st exon or until stop codon of 2nd exon was counted.

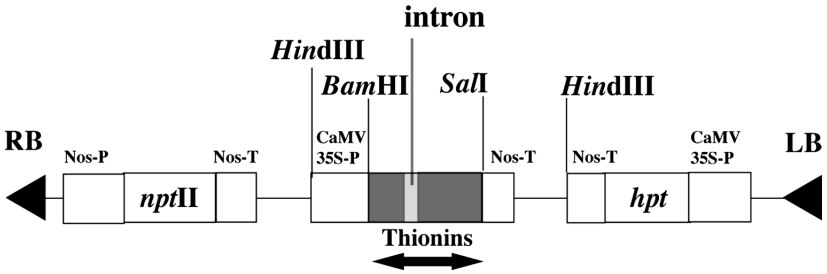

Figure 1. Transfer DNA region of the binary plasmid vectors used in this study. Each thionin genes (*BoT5*, *BoT6*, *NoT2*, *BvT2*, or *BvT4*) was inserted into between cauliflower mosaic virus 35S transcript promoter (CaMV 35SP) and nopaline synthase terminator (*nos*T) of pEKH2 binary vector. Hygromycin phosphotransferase (*hpt*) gene is driven by CaMV 35SP, whereas the gene for neomycin phosphotransferase (*npt*II) by *nos*P. Double arrows show the thionin gene used as the probe for Southern blot analysis. RB and LB, Right and left border sequences of the T-DNA region, respectively. Recognition sites of restriction enzymes are also indicated

A

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Bo    MAKVASIVALLFPALVIFAAFEAPTMVEAQKLCERPSGTW--SGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC     80
Rs    MAKFASIIVLLFAALVVFSAFEAPTMVEAQKLCQRPSGTW--SGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC     80
Ew    MAKFASIIALLFAALVLFSAFEAPSMVEAQKLCEKSSGTW--SGVCGNNNACKNQCINLEGARHGSCNYIFPYHRCICYFPC     80
NoT2  MAKFASIITLIFAALVLFAAFEAPIMVVAQRLCEKPSGTW--SGVCGNSNACKNQCINLEGARHGSCDYVFPAHKCICYFPC     80
BoT5  MAKFASIIVLLFA--------EAPTMVEAQKLCERPSGTW--SGVCGNNNGCKNQGIRLEKARHGSCNYVFPAHKCICYFPC     72
BoT6  MAKFASIVALLFSALVIFAAFEAPTMVEAQKLCERPSGTW--SGVCGNNNACKNQCIRLEKARHGSCNYVFPAHKCICYFPC     80
BvT2  MAKFASIITLIFAALVLFAAFEAPIMVVAQRLCEKPSGTW--SGVCGNSNACKNQCINLEGARHGSCNYVFPAHKCICYFPC     80
BvT4  MAKFASIITLLFAALVLFAALEASALRGGKR-CEKRNSSTSFSGVCQYDNACMNQCINLEGAQDGKCNNAVPTPKCICYFPCFPNS  85
      *******  * *         **         *   *     *      * * ** * ** *  * **   *   *******
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B

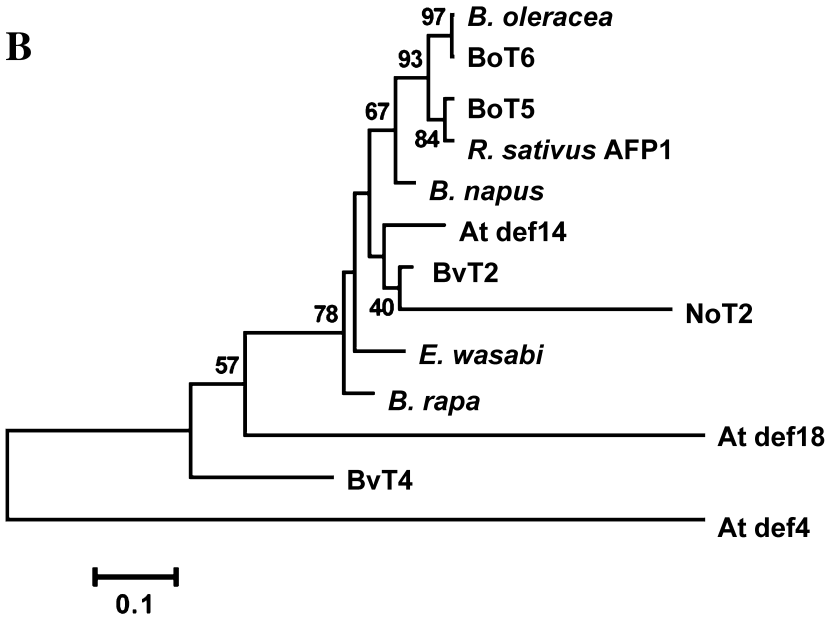

Figure 2. Comparison of deduced amino acid sequences among five thionins from three Brassicaceae species. (A) Alignment of deduced amino acid sequences among five thionins from three Brassicaceae species. Five isolated thionins: BoT5, BoT6 (*Brassica oleracea*), NoT2 (*Nasturtium officinale),* BvT2, and BvT4 (*Barbarea vulgaris*), and three other known thionins were aligned. Bo: defensin (Genbank accession No. CAC37558) in *Brassica oleracea*. Rs: antifungal protein 1 (RSU18557) in *Raphanus sativus*. Ew: gamma-thionin (AB012871) in *Eutrema wasabi*. A big arrowhead corresponds to the intron position of thionin gene. Arrowheads and asterisks indicate seven conserved Cysteine "C" residues and identical amino acids among thionins, respectively. (B) Phylogenetic relationships of amino acid sequences among thionins in Brassicaceae species. At def4: defensin-like protein 4 (NM_126272) in *Arabidopsis thaliana*. Atdef14: defensin-like protein 14 (NM_128160) in *Arabidopsis thaliana*. Atdef18: defensin-like protein 18 (NM_104375) in *Arabidopsis thaliana*. *B. napus*: antifungal protein (BNU59459) in *Brassica napus*. *B. olearacera*: defensin (AJ311046) in *Brassica oleracea*. *B. rapa*: defensin (AF528180) in *Brassica rapa*. *E. wasabi*: gamma-thionin in *Eutrema wasabi*. *R. sativus* AFP1: antifungal protein (AFP) 1 in *Raphanus sativus*. Phylogenetic relationships were constructed by the Neighbor-Joining option of MEGA 4.0, using 1000 bootstrap replicates.

amino acid sequences of their middle parts were not homologous to one another (Figure 2A). All thionins shared seven conserved cysteine "C" residues, and one glutamic acid "E" at which an intron is located. These cystein residues covalently link to form tertiary structure *via* disulfide bridges (Hendrickson and Teeter 1981) and are involved in the antifungal activity (Bohlmann et al. 1988; Wada et al. 1982). BoT5 had a deletion of eight amino acids in the N-terminal region and BvT4 contained an extension of four amino acids at the C-terminus.

The sequence similarity of thionin was analyzed using MEGA4 program (Tamura et al. 2007). Four thionins, BoT5, BoT6, NoT2, and BvT2, claded with *Raphanus sativus* anti-fungal protein 1 (AFP1) and *Eutrema wasabi* gamma-thionin (Figure 2B), of which antifungal activities were earlier proven by the genetic transformation studies (Kanzaki et al. 2002; Khan et al. 2006; Ntui et al. 2011; Terras et al. 1995). In contrast, amino acid sequence of BvT4 protein diverged from those of wasabi gamma-thionin, and four other thionins (Figure 2B).

Genomic sequences of five thionin genes were respectively integrated into binary vector pEKH2 between kanamycin and hygromycin selection marker cassettes (Figure 1). *A. tumefaciens* strain EHA105 by freeze-thaw method (Holsters et al. 1978; Hood et al. 1986). Transgenic plants of potato cultivar 'Waseshiro' were generated by *Agrobacterium*-mediated transformation using the binary vector containing each thionin gene as described by Khan et al. (2006, 2008). One month after the inoculation, shoots were regenerated from green nodular calli, which appeared on the surfaces of the tuber discs. Transformation efficiencies were 25–50% in this study. Regenerated shoots showed normal morphology. Rooted transgenic plants were used for molecular analysis and fungal disease resistance assay.

Southern blot, Reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis of potato plants were described previously (Khan et al. 2006, 2010; Ntui et al. 2010). Integrations of the thionin transgenes into potato genomes were confirmed by Southern blot analysis. *Sal*I-digested genomic DNAs (20 μg) of transgenic and non-transgenic plants were hybridized with DIG-labeled probes of the thionin genes (Figure 3A). Although non-transgenic plant had no signal, all transgenic plants harboring either *BoT5*, *BoT6*, *NoT2*, *BvT2*, or *BvT4* transgene showed various banding patterns, with integration of one to five transgenes in the genomes. Transgenic plants containing a single- or low-copy transgenes were selected for further analyses.

For RT-PCR analysis, total RNAs were extracted as described by Krapp et al. (1993). First strand cDNAs were synthesized from 1 μg of total RNA in a 20 μl

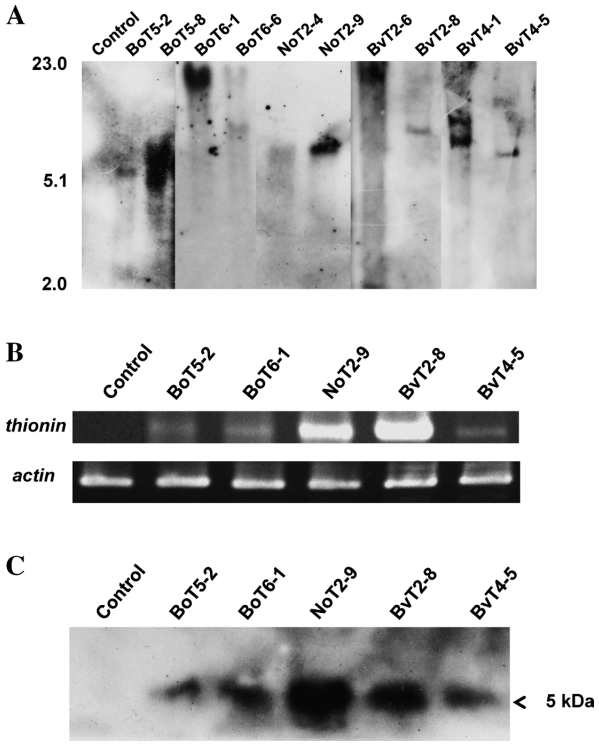

Figure 3. Molecular analyses of transgenic potato plants. (A) Integration of thionin genes into genomic DNA of transgenic potato plants. Genomic DNA's were digested with *Sal*I. Lanes: non-transformed control plant (Control), independent transgenic potato lines harboring *BoT5*, *BoT6*, *NoT2*, *BvT2*, or *BvT4* thionin gene, respectively. (B) RT-PCR analyses of *thionin* transcripts in transgenic plants (upper row). Lanes: non-transformed control plant (Control), independent transgenic potato lines harboring each thionin gene. Actin gene was used as the loading control (lower row). (C) Western blot analysis of thionin protein in transgenic potato plants. Lanes: non-transformed control plant (Control), independent transgenic potato lines harboring each thionin gene.

reaction volume using SuperScript Transcriptase III (Life Technologies, USA). RT-PCR were performed using Cthio5P 5′-GGATCCATGGCGAAATTTGCGTCG-3′ and Thio3P as follows: 25 cycles of 94°C for 1 min (denaturation), 54°C for 1 min (annealing) and 72°C for 1 min (elongation). Amounts of the amplified PCR products were much higher in NoT2-9 and BvT2-8 than in BoT5-2, BoT6-1, and BvT4-5 transgenic plants (Figure 3B). Actin cDNA fragment was amplified in parallel, as a control, using a pair of primers based on rice actin gene, RTAct1: 5′-GAAGGATCTATATGGCAACATCG-3′ and Act2: 5′-ATCCACATCTGCTGGAATGTG-3′ as follows: 30 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing) and 72°C for 2 min (elongation). The amplified PCR products were fractionated through 1% agarose gel.

Protein products (*ca.* 5 kDa) of introduced thionin genes were analyzed by western blot analysis using a primary antibody against gamma-thionin of *Eutrema wasabi* (Figure 3C). Transgenic line NoT2-9 showed the strongest signal than the other four lines. Furthermore,

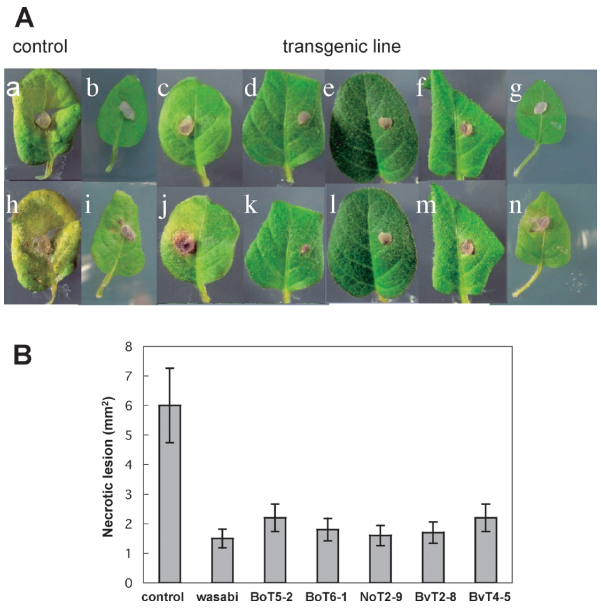

Figure 4. Anti-fungal resistance assay using detached leaves of the non-transformed and transgenic potato plants expressing each thionin gene. (A) Photographs were taken at 3 days (upper row) and one week (lower row) after the inoculation with blocks of agar containing conidia of *Botrytis cinerea*, non-transformed plant (a, h), transgenic potato expressing wasabi thionin gene (positive control: b, i), transgenic potato line BoT5-2 (c, j), BoT6-1 (d, k), NoT2-9 (e, l), BvT2-8 (f, m), BvT4-5 (g, n). (B) Detached leaves of the transgenic and non-transformed control were inoculated with blocks of agar containing conidia of *B. cinerea*. Average area (mm^2) of the necrotic lesions on 10 detached leaves was measured one week after inoculation.

line BvT2-8 had a higher amount of mRNA than line NoT2-9, whereas the protein level of line BvT2-8 was lower than that of line NoT2-9. Non-transformed control plant did not show any positive signal, indicating that proteins of potato did not cross-reacted with the antibody against wasabi gamma-thionin.

Fungal resistance of transgenic potato plants expressing thionin genes isolated from Brassicaceae species was evaluated against the phytopathogenic fungus, gray mold (*Botrytis cinerea*) as described by Khan et al. (2010) with a slight modification. Briefly, mycelium of *Botrytis cinerea* were grown on Petri dishes containing potato-dextrose agar (PDA, Difco). A block of agar containing conidia, was concentrically excised from the fungal medium using a Cork borer (1.0 cm in diameter) and placed on a leaf of transgenic or control potato plants. The inoculated leaves were maintained in a humid container at room temperature under a 16 h light/8 h dark regime. Ten leaves from each transgenic plant were used for the disease resistance assay, and the assay was repeated twice. Photographs of lesions were taken 3 days and one week after inoculation. Transgenic potato expressing gamma thionin gene of *Eutrema wasabi* was provided by Dr. Raham S. Khan, Graduate School of Horticulture, Chiba University, Japan. This plant was used as the control for disease resistance assay.

The inoculated leaves of non-transformed control plant showed necrotic lesions up to 60–80% of total leaf area one week after inoculation (Figure 4A). Furthermore, the area of necrotic lesions observed in the transgenic plants was about 30%, which was significantly smaller compared to that of the control plants (Figure 4B).

The transgenic potato plants containing single- or low-copy transgenes were selected by Southern blot analysis (Figure 3A). Although the thionin genes were expressed under the control of CaMV 35S promoter, two transgenic plants (NoT2-9, BoT2-8) showed higher amounts of mRNA expression (Figure 3B) and protein products (Figure 3C) than the other three transgenic plants (BoT5-2, BoT6-1, and BvT4-5). These results indicated that the expression levels of mRNA and protein from thionin genes were different among these five transgenic plants. And amino acid sequence of BvT4 protein differed from those of other thionins (Figure 2A). In spite of these differences, disease resistance assay showed that all five transgenic plants had similar levels of resistances against *B. cirenea* (Figures 4), which were also similar to those of the transgenic control plant expressing gamma-thionin gene of *Eutrema wasabi*. Although the level of resistance has often been associated with the amount of protein expression (Ntui et al. 2010), our result does not show such correlation. Therefore, further research will be necessary to reveal the relationship between the amount of thionin and resistance to fungal pathogens. In this study, genomic thionin genes containing an intron were introduced in the genomes of potato plants whereas most transgenic researches have been performed using cDNA clone of thionin gene as transgene. Ohta et al. (1990) reported that *GUS* gene containing an intron of caster bean catalase gene showed enhanced expression of the reporter. However, in this study, it was difficult to evaluate the effect of the intron on the accumulation of thionin protein from the transgene because thionin was too small (5 kDa) to retain on the PVDF membrane.

Plants contain various kinds of small cystein-rich proteins, which are classified into several families, such as thionin, snaking, defensin-like, hevein-like, and lipid transfer protein, on the basis of primary structure (Castro and Fontes 2005; Garcia Olmedo et al. 1998). In *Arabidopsis thaliana*, one of the Brassicaceae species, contains more than hundred genes encoding defensin-like proteins. Although potato did not contain immunological positive protein against antibody of wasabi gamma-thionin (Figure 3C), it probably has another family of anti-microbial proteins. Epple et al. (1997) described that over-expression of endogenous thionin gene in *Arabidopsis* under the control of CaMV 35S promoter enhanced resistance against *Fusarium oxysporum*. In their study, there were no correlations between expression levels of thionin transgene and disease indexes (Figure 3B, 4). Why would transgenic

plants expressing the endogenous thionin show resistance against pathogenic fungi in spite of the amount of thionin? One plausible explanation is that spatiotemporal changes on expression pattern of thionin gene may be responsible for such defense system. However, detail functions of thionin on the defense mechanism remains to be understood.

We have successful cloned and integrated five novel thionin genes into the genomes of potato. Transgenic potato plants expressing each thionin gene demonstrated similar levels of resistance to *B. cinerea*. However, further researches will be necessary to produce transgenic plants of potato with higher resistance to *B. cinerea* and to test the resistance to other pathogenic fungi and bacteria such as *Phytophthora infestans*, *Alternaria solani*, and *Erwinia carotovora*.

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