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Protein Note

Pathogenesis-related protein 1 homologue is an antifungal protein in *Wasabia japonica* leaves and confers resistance to *Botrytis cinerea* in transgenic tobacco

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Abstract An antimicrobial protein was purified from leaves of *Wasabia japonica* L., and designated as WjAMP-2. WjAMP-2 strongly inhibited growth of four phytopathogenic fungi. The deduced amino acid sequence from WjAMP-2 cDNA showed a homology with pathogenesis-related protein 1 (PR-1) from several higher plants. Expression of *WjAMP-2* was induced by inoculation with fungal pathogens and with treatments by salicylic acid, but not by methyl jasmonate. Overexpression of *WjAMP-2* in tobacco plants conferred resistance against *Botrytis cinerea*. These results suggest that WjAMP-2 (PR-1) is an antifungal protein with a defensive role against phytopathogenic fungi in wasabi plants.

Key words: Antimicrobial protein, pathogenesis-related protein 1, transgenic tobacco, *Wasabia japonica*.

Plants are endowed with a wide variety of defense mechanisms that protect them from attack by pathogenic microorganisms. Active defense mechanisms include hypersensitive cell death (Tomiyama 1967; Levine et al. 1994), production of phytoalexins (Kuc 1972), expression of pathogenesis-related proteins (Mauch and Staehelin 1989), oxidative burst (Baker and Orlandi 1995), cross-linking of cell wall glycoprotein (Bradly et al. 1992; Brisson et al. 1994) and lignification (Vance et al. 1990). Many plant proteins with antimicrobial activity have also been identified. For example, 14 families of pathogenesis-related (PR) protein (PR-1–14) were recognized and classified (Van Loon and Van Strien 1999). In *Arabidopsis thaliana*, the expression of *PR-1*, *PR-2* and *PR-5* is required for increased protection against *Pernospore parasitica* and *Pseudomonas syringae*. It has also been reported that induction of *PDF1.2*, *PR-3* and *PR-4* is associated with induced resistance to *Alternaria brassicicola*, *Botrytis cinerea* and *Erwinia carotovora* (Thomma et al. 2000; Van Loon and Van Strien 1999). Moreover, ectopic over-expression of *PR* genes enhances resistance to plant pathogens (Evans and Greenland 1998). Thus, expression of PR protein is associated with the induction of acquired resistance.

Wasabi (*Wasabia japonica*) is widely used in Japan, both as a foodstuff and as wrapping material to protect food from putrefaction. Secondary metabolites of wasabi such as wasalexin and 6-methylsulfonylhexyl isothiocyanate reportedly possess antifungal and antibacterial activity, respectively (Pedras et al. 1999; Ono et al. 1998). However, little is known about the relationship between antimicrobial proteins and the defense responses of wasabi plants. We previously reported that one of the major antimicrobial proteins in wasabi leaves, *Wasabia japonica* antimicrobial protein-1 (WjAMP-1), is the C-terminal domain of hevein-like protein (Kiba et al. 2003). The plant defensin gene *WT1* was also cloned from wasabi plants and studied for its antimicrobial activity using the Potato virus X (PVX) vector system (Saitoh et al. 2001) and transgenic plants (Kanzaki et al. 2002; Khan et al. 2006; Sjahril et al. 2006). These studies suggest that wasabi might be a good source for isolation of novel antimicrobial proteins.

In this paper, we describe a novel antimicrobial protein, WjAMP-2, purified from wasabi leaves, with a strong inhibitory effect on the growth of a broad group of plant fungal pathogens. We also discuss the role of

Abbreviations: BSA, bovine serum albumin; DIG, digoxigenin; HPLC, high performance liquid chromatography; PR, pathogenesis related; RACE, rapid amplification of cDNA ends.

The nucleotide sequence of *WjAMP-2* (PR-1) reported in this paper has been submitted to DDBJ, EMBL and Gene Bank under accession number AB271488.

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antimicrobial protein in the defense responses of wasabi plants.

Wasabia japonica cv. Mazuma was kindly provided by Mr. S. Sasaki (Miyamori wasabi bio-company, Tono city, Iwate, Japan). *Botrytis cinerea* strain S1 and *Fusarium solani* strain IFS-1 were grown on potato dextrose agar at 25°C for two weeks under white fluorescent lighting (Kiba et al. 2003). *Alternaria alternata* apple pathotype strain IAAP-1 and *Magnaporte grisea* strain P2 were grown at 25°C for two weeks under blue fluorescent lighting on potato dextrose agar and oatmeal agar, respectively (Kiba et al. 2003).

Antimicrobial protein was isolated according to the method of Kiba et al. (2003). One kilogram of mature wasabi leaves was homogenized with homogenizer (ULTRA-TURRAX T25, JANKE & KUNKEL IKA-Labortechnik GMBH&CO. KG) in extraction buffer as described previously (Kiba et al. 2003). Assay for growth inhibition activity was spectrophtometrically estimated as described by Cammue et al. (1992) with the fungal pathogen *B. cinerea*. The extract was then centrifuged for 30 min at 32000×*g*. The ammonia sulfate-precipitated fraction between 30 to 70% saturation, which had strong antifungal activity, was obtained, and the unbound protein fraction (basic protein fraction) obtained using an anion exchange Q-sepharose column (Amersham Pharmacia, Buckinghamshire, England) equilibrated with 50 mM Tris-HCl (pH 9.0). Basic protein fraction was applied to a cation exchange SP-sepharose column (Amersham Pharmacia) equilibrated with 50 mM MES buffer (pH 6.0), and eluted with 0.5 M NaCl in the same buffer. Much of antifungal activity was detected in eluted fraction, but not in unbound fraction. The eluted fraction was applied to reverse phase high-performance liquid chromatography using a TSKgel phenyl-5PW RP column (Toso, Tokyo, Japan) in equilibrium with 0.1% TFA. Proteins were eluted with a linear gradient of 0–50 % solvent B (0.1% TFA in 100% acetonitrile) in solvent A (0.1% TFA in deionized water) at a flow rate of 1 ml min^{-1} for 200 min and collected every 1 min. We obtained two protein fractions showing strong antifungal activity, which were designated as WjAMP-1 (*Wasabia japonica* antimicrobial protein-1; Kiba et al. 2003) and WjAMP-2 (Figure 1), respectively. The protein concentration was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

As shown in Figure 1, the purified fraction indicated by the arrow strongly inhibited the growth of *B. cinerea* (WjAMP-2). Analysis of the fraction by SDS-PAGE clearly showed a single band of about 15 kDa. Microscopic observation showed that WjAMP-2 inhibited both spore germination and hyphal growth of *A. alternata*. Moreover, WjAMP-2 inhibited growth of four kinds of fungi, *A. alternata*, *B. cinerea*, *F. solani* and *M. grisea*. The concentrations required for 50% inhibition of growth (IC_{50}) of these fungi were 14.5, 11.0, 6.9 and 60 $\mu g\,ml^{-1}$, respectively. Antifungal activity was scarcely affected in the concomitant presence of cations (Table 1).

To further characterize WjAMP-2, the purified protein was digested with trypsine at 37°C for 1 h according to the manufacturer's protocol (Promega, Wisconsin, USA) and amino acids up to 12 th residue was determined as follows: Leu-Ile-His-Ser-Gly-Gly-Pro-Tyr-Gly-Glu-Asn-Leu (HP G1005A, Takara Shuzo customer service, Shiga Japan). Based on the internal amino acid sequence of

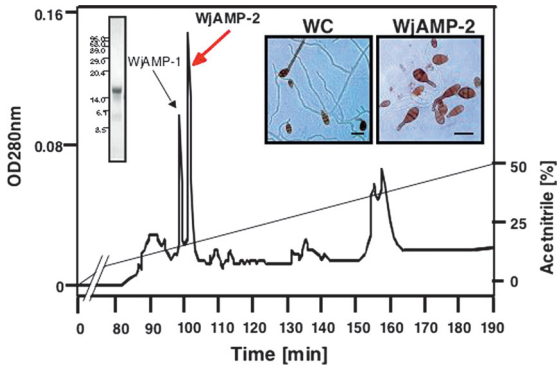

Figure 1. Purification of WjAMP-2 by reverse-phase HPLC. The protein fraction partially purified using a cation exchange column was separated with a reverse-phase column as described in the text. The arrows correspond to WjAMP-1 (Black arrow) and WjAMP-2 (red arrow). Inset figures show SDS-PAGE analysis of WjAMP-2 and microscopic observations of the growth of *A. alternata* 24 h after the start of culture in the absence (WC) and presence of WjAMP-2 at a concentration of 10 $\mu g\,ml^{-1}$. Bar represents 10 μm.

Table 1. Antifungal activity of WjAMP-2 in presence of different cations

IC_{50} [$\mu g\,ml^{-1}$]						
		Medium A supplimented with				
Medium A	Medium B	50 mM K^+	50 mM Na^+	50 mM NH^{4+}	50 mM Mg^{2+}	50 mM Ca^{2+}
14.5	22.3	17.2	19.2	22.1	19.6	18.9

Protein concentrations required for 50% growth inhibition (IC_{50}) after 24 h of incubation, determined from dose-response curve in the absence or presence of cations.

IC_{50} values for the protein were determined on *Botrytis cinerea* in medium A, B and medium A sopplimented with 5 mM $CaCl_2$, 50 mM KCI, 5 mM $MgCl_2$, 50 mM NaCl or 50 mM NH_4Cl.

[a] Medium A: Synthetic growth medium

[b] Medium B: medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCI.

WjAMP-2, the following primer was designed: 5′-Y(C/T)TIATH(A/C/T)CAY(C/T)K(G/T)SIGGIGGICCI-TA-3′ (WjAMP2RT), for reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed with a High Fidelity RT-PCR kit (Takara Shuzo). For isolation of complete WjAMP-2 cDNA, a modified rapid amplification of cDNA end (RACE) method (5′-RACE) was performed. Based on the internal nucleotide sequences of the RT-PCR product, the following primer was designed: 5′-TATGCCAGATAAGTCGCTGCTTCC-3′ (WjAMP2-5). Using the nucleotide sequences of the products of RT-PCR and 5′-RACE, a specific primer, 5′-GTCACAAAAAAGCTTGAAAATAGCC-3′, (WjAMP2S), was synthesized to generate full-length cDNA. Full-length cDNA was amplified with WjAMP2S and M13M4 (5′-GTTTTCCCAGTCACGAC-3′) and cloned into the pCR2.1 vector (Invitrogen, California, USA). DNA sequencing was performed using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan) and Applied Biosystems 377 Automated Sequencer. Sequence alignment and analyses were carried out using DNASIS (version 3.6; Hitachi, Tokyo, Japan) and the BLAST network service of the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990).

The full-length 679-bp cDNA corresponding to *WjAMP-2* contained an open reading frame (ORF) encoding a polypeptide of 161 amino acids, including the same amino acid sequence of internal amino acids determined by peptide sequencing (Figure 2). A protein database search showed 79, 71, 70, 59 and 55% amino acid homology with PR-1s from *Arabidopsis thaliana* (P33154), *Brassica napus* (AAB06458), Zea mays (A33155), *Nicotiana tabacum* (X05959) and *Glycine max* (AF136636), respectively. Subcellular localization of WjAMP-2 was predicted to be extracellular using the PSORT program. Predicted isoelectric point of mature form of WjAMP-2 (putative signal peptide 1 to 19aa) was also calculated as 8.51.

Numerous researchers have attempted to assess the function of PR-1 in plants. For example, Niderman et al. (1995) reported that PR-1 from tobacco showed fungicidal activity against *Phytophthora infestans*, while PR-1 protein from broad bean exhibited strong differentiation-inhibiting activity towards *Uromyces fabae* (Rauscher et al. 1999). The present study also showed that WjAMP-2 (PR-1) possesses antifungal activity against a broad range of phytopathogenic fungi. As far as we know, this is a first report about antifungal activity against broad range of phytopathogenic fungus, since previous repots mainly focused on antifungal activity against limited fungus. Therefore, PR-1 is also thought to function in plant defenses against phytopathogenic fungi in wasabi plants.

In recent years, many plant proteins with antimicrobial activity have been identified (Broekaert et al. 1997). These antimicrobial proteins contain various characteristic features responsible for their antimicrobial activity. For example, chitin-binding protein contains a chitin-binding domain that binds to chitin, a major cell wall component of fungi (Raikhel and Lee 1993), while PR-4, hevein-like protein and win-type protein have a barwin structure (Linthorst 1991). The antimicrobial activity of cysteine-rich antimicrobial proteins such as plant defensin, hevein-like protein and thionine is thought to be controlled by specific structures formed by inter-cysteine disulfide bonds (Broekaert et al. 1995). These proteins are thought to target membrane structures of microorganisms, since these types of antimicrobial activity are quite sensitive to the presence of cations (Garcia-Olmedo et al. 1995; Kiba et al. 2003; Osborn et al. 1995). Though WjAMP-2 (PR-1) contains cystein residues conserved among PR-1 from various plant species (Figure 2), the antifungal activity of WjAMP-2 was scarcely affected by the presence of cations (Table 1). Therefore, WjAMP-2 is thought to show antifungal activity controlled by a mechanism different from plant defensin, thionin, LTP and hevein-like protein. Thus, the molecular mechanism responsible for the antifungal activity of PR1 remains to be elucidated.

Southern blot analysis was carried out as described previously (Kiba et al. 2003) using a digoxigenin (DIG)-labeled cDNA probe created with a PCR DIG labeling Kit (Roch, Mannheim, Germany) using WjAMP2S and WjAMP2A (5′-TTAGTAAGGTTTCTGGTTCAC-3′) as primers. Hybridization signals were detected using alkaline phosphatase-conjugated anti-DIG antibody and CSPD (Roche). Southern blot hybridization analysis with the cloned cDNA used to probe the wasabi genome indicated, at most, seven copies of *WjAMP-2* gene homologues (Figure 3A). This result coincided with those of a previous report on tobacco plants (Cornelissen et al. 1987).

Northern blot analysis of RNA isolated from young and mature leaves, petioles, stems and roots were carried out according to the method of Kiba et al. (2003). *WjAMP-2* mRNA was most abundant in petioles (Figure 3B). A relatively low level of expression was detected in young and mature leaves, stems and roots. The level of *WjAMP-2* RNA expressed in mature leaves of *W. japonica* inoculated with spore suspension (1×10^5 spores ml^{-1}) of incompatible fungal pathogen, *B. cinerea* or *A. alternata*, significantly increased 24 and 48 h after inoculation (Figure 3D). Treatment of leaves with 2 mM salicylic acid (SA) also strongly induced *WjAMP-2* mRNA synthesis, but no significant effect of 50 μM methyl jasmonate (MeJA) was observed (Figure 3D). Both chemical treatments did not show any visible damages on wasabi leaves. These results suggest that expression of *WjAMP-2* is induced by fungal infection

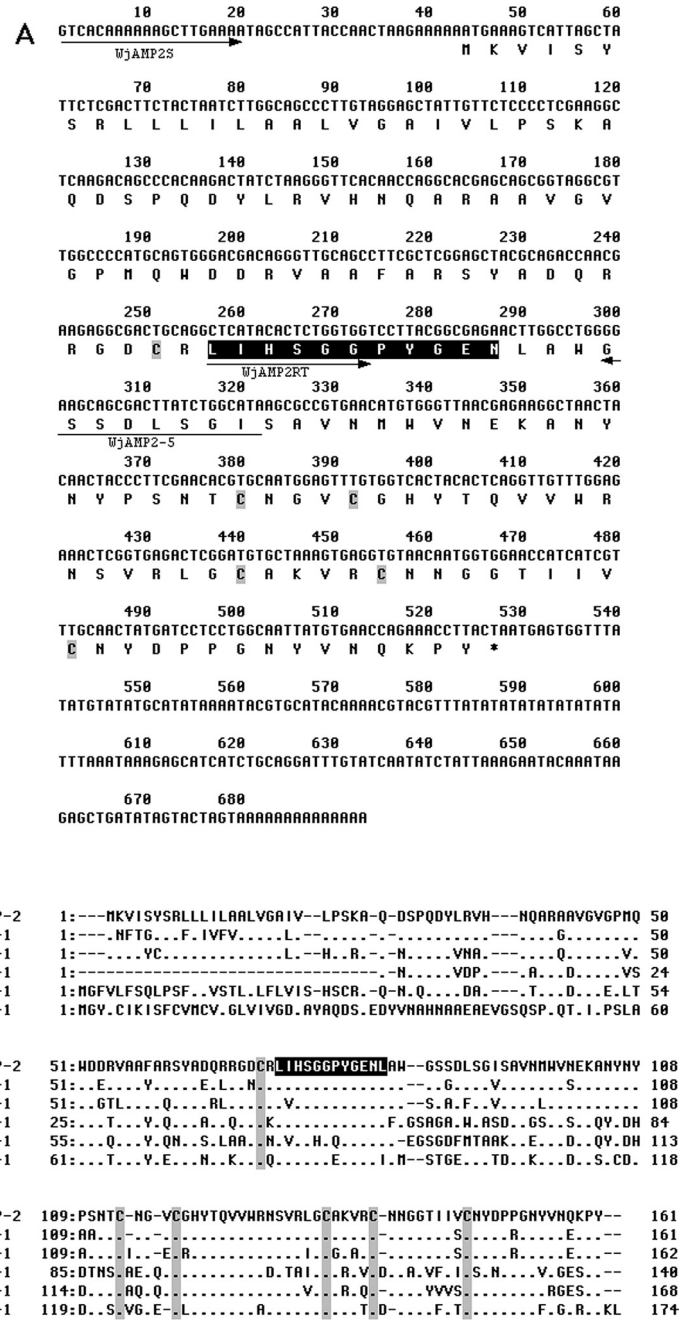

Figure 2. Nucleotide and deduced amino acid sequences of *WjAMP-2* cDNA. (A) The peptide sequence obtained from the purified protein is shown in black box. Conserved cysteine residues are shown in grey boxes. Arrows showed primers used to isolate full length cDNA of *WjAMP-2*. (B) Multiple alignment of WjAMP-2 with PR-1 proteins from *Arabidopsis thaliana* (AtPR-1; P33154), *Brassica napus* (BnPR-1; AAB06458), *Zea mays* (ZmPR-1; A33155), *Nicotiana tabacum* (NtPR-1; X05959) and *Glycine max* (GmPR-1; AF136636). The peptide sequence obtained from the purified protein is shown in black box. Conserved cysteine residues are shown in gray box.

and is regulated by a SA-dependent signal transduction pathway. Because *WjAFP2* belongs to multiple copy gene family as shown by Southern blot analysis, it is also necessary to reveal the contribution of the each *WjAFP-2* homolog to the induced expression in future studies.

Plants are capable of differentially activating distinct defense pathways. In tobacco, for example, SA is a critical signaling molecule leading to the expression of acidic types of PR proteins (Guo et al. 2000; Ohashi and Ohshima 1992). PR-1 is a dominant group of PR proteins induced by pathogen inoculation and SA treatment, and is commonly used as a marker for systemic acquired resistance (van Loon and van Strien 1999). Our present data also shows that *WjAMP-2* is regulated by a SA-dependent pathway in a similar manner to tobacco plants (Figure 3). In addition, JA also mediates expression of basic types of PR gene such as *PR-1b*, *PR-5* and *PR-6* (Niki et al. 1998). We also

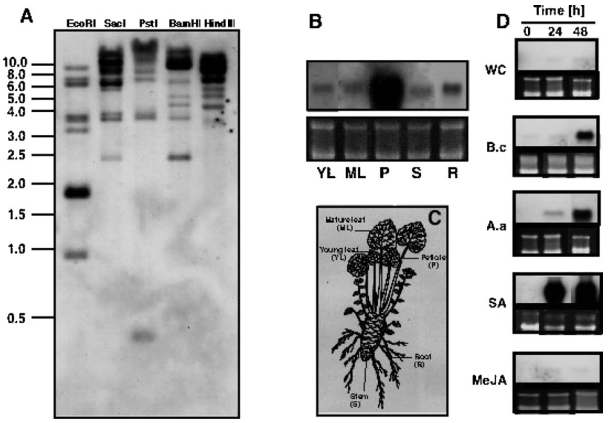

Figure 3. Southern and northern blot analysis of *WjAMP-2*. (A) Genomic DNA isolated from *W. japonica* (10 μg) was digested with *Eco*RI, *Sac*I, *Pst*I, *BamH*I and *Hind*III then probed with a DIG-labeled cDNA insert of *WjAMP-2*. (B) Total RNA was isolated from young leaves (YL), mature leaves (ML), petioles (P), stems (S) and roots (R) as shown in (C). (D) Total RNA was isolated from water-treated mature leaves (WC) or mature leaves inoculated with *B. cinerea* (Bc) or *A. alternata* (Aa), or treated with methyl salicylic acid (SA) or jasmonate (MeJA). The RNA was hybridized with a DIG-labeled cDNA insert of *WjAMP-2*.

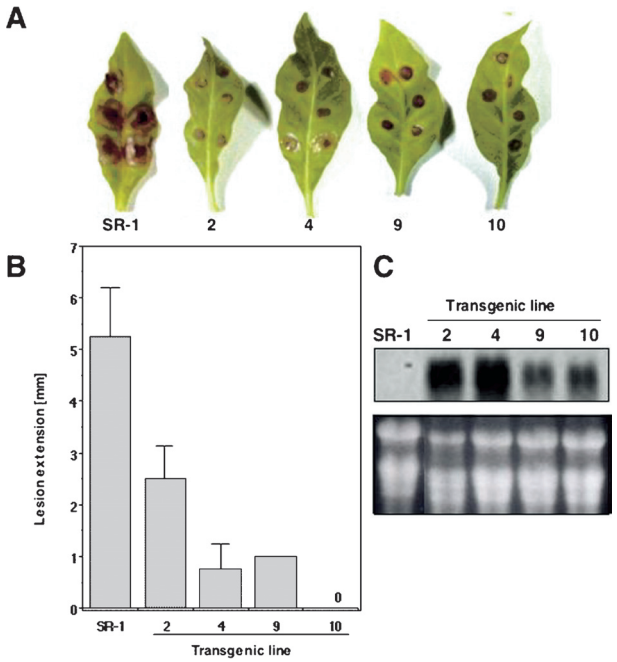

Figure 4. Enhancement of disease resistance against the fungal pathogen *Botrytis cinerea* in transgenic tobacco. (A) Lesions caused by the fungus 5 days after inoculation in non-transformed control (SR-1) and *WjAMP-2*-expressing T_2 transgenic plants (nos. 2, 4, 9 and 10). (B) Lesion extension 5 days after inoculation. Values represent the mean with standard deviation (SD) of results from triplicate experiments. (C) Expression of the *WjAMP-2* gene in transgenic tobacco plants. Total RNA was isolated from fully expanded leaves of untransformed control (SR-1) and T_2 *WjAMP-2* gene-transformed plants (nos. 2, 4, 9 and 10). Equal loading of RNA was verified by staining with ethidium bromide (bottom).

previously reported that expression of *WjAMP-1* (the C-terminal domain of hevein-like protein) is stimulated by treatment with MeJA, but not with SA at same conditions (Kiba et al. 2003). These findings suggest that SA- and JA-regulated signal transduction pathways play crucial roles in defense responses against plant pathogens in wasabi plants.

To clarify the role of *WjAMP-2* on plant immunity against phytopathogenic fungi, *WjAMP-2* was overexpressed in tobacco plants. The ORF of the *WjAMP-2* gene was cloned between the CaMV35S promoter and nopaline synthase terminator of pBIS221S (Kiba et al. 2005) using *Bam*HI and *Sal*I sites. The pBIS221SWjAMP-2 was then cut with *Sse* I and cloned into a binary vector, pEKB (Mishiba et al. 2005), resulting in pEKB-WjAMP-2. The construct was introduced into *Agrobacterium tumefacien*s EHA105 by electroporation (MicroPulser: Bio-Rad, Tokyo, Japan) then tobacco plants (*N. tabacum* cv. SR-1) aseptically grown from seeds for about 1 month were transformed via an *Agrobacterium tumefaciens*-mediated leaf disc procedure (Horsch et al. 1985) and selected using 5 $\mu g\,ml^{-1}$ of bialaphos (Meiji Seika, Tokyo, Japan) as the selection reagent. Four transgenic T_2 plant lines homozygous for bialaphos resistance and showing a high level of *WjAMP-2* expression were selected by Northern blot analysis using *WjAMP-2* cDNA. There were no visible morphological changes in transgenic lines. Spores of *B. cinerea* were resuspended in potato glucose liquid medium and adjusted to 1×10^8 spores ml^{-1} then filter paper (8 mm^2) dipped in the spore suspension was placed 5 spots onto detached fully expanded leaves from 4- to 5-week-old transgenic and wild-type tobacco plants. Three leaves from 3 independent plants were used for this experiment. They were kept in a moistened box at 25°C under continuous illumination at a light intensity of 30 $\mu Em^{-2}s^{-1}$ then 5 days after inoculation lesion size was measured. Results were represented as lesion extension (mm), which were values subtracted the diameter of filter paper from total lesion diameters.

As shown in Figure 4, typical lesions were observed extending about 5 mm in detached leaves of wild-type tobacco plants 5 days after inoculation with *B. cinerea* spores. In contrast, lesions were significantly reduced in the 4 transgenic lines. Although lesion extension was slightly observed in transgenic lines by prolonged observation (10 days after inoculation), significant reduction of lesion size was observed in transgenic lines (data not shown). Transgenic lines that failed to express *WjAMP-2* expression showed no enhancement of disease resistance against *B. cinerea* (data not shown). These results suggested that overexpression of WjAMP-2 suppresses disease development of *B. cinerea*. Therefore, WjAMP-2 may have an antifungal activity *in planta*. A previous report showed that constitutive high-level expression of PR-1 in transgenic tobacco results in

tolerance to infection by two oomycete pathogens, *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotianae* (Alexander et al. 1993). Taken together, these findings suggest that WjAMP-2 (PR-1) might play a crucial role in defense against a broad range of filamentous fungi in wasabi plants. *WjAMP-2* gene might be useful for genetic engineering of enhanced disease resistance.

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References

Alexander D, Goodman RM, Gut-Rella M, Glascock C, Weymann K, Friedrich L, Maddox D, Ahl-Goy P, Luntz T, Ward E, Ryals J (1993) Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc Natl Acad Sci USA* 90: 7327–7331

Altschul SF, Gish W, Miller W, Liptman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215: 403–410

Baker CJ, Orlandi EW (1995) Active oxygen in plant pathogenesis. *Annu Rev Phytopathol* 33: 299–321

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254

Bradly D, Kjellbom P, Lamb CJ (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel rapid defense response. *Cell* 70: 21–30

Brisson LF, Tenhaken R, Lamb C (1994) Function of oxidative cross-linking of cell wall structural proteins in plant disease resistance. *Plant Cell* 6: 1703–1712

Broekaert WF, Cammue BPA, De Bolle MFC, Thevissen K, de Samblanx GW, Osborn RW (1997) Antimicrobial peptides from plants. *Cri Rev Plant Sci* 16: 297–323

Broekaert WF, Terras FRG, Cammue BPA, Osborn RW (1995) Plant defensins: Novel antimicrobial peptides as components of the host defense system. *Plant Phisiol* 108: 1353–1358

Cammue BPA, De Bolle MFC, Terras FRG, Proost P, Van Damme J, Rees SB, Vanderleyden, J, Broekeart WF (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J Biol Chem* 267: 2228–2233

Cornelissen BJC, Horowitz J, van Kan JAL, Goldberg KR, Bol JF (1987) Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. *Nucl Acid Res* 15: 6799–6811

Evans IJ, Greenland AJ (1998) Transgenic approaches to disease protection: applications of antifungal proteins. *Pestic Sci* 54: 353–359

Garcia-Olmedo F, Molina A, Segura A, Mooreno M (1995) The defensive role of non specific lipid-transfer proteins in plants. *Trend Microbiol* 3: 72–75

Guo A, Salih G, Klessig DF (2000) Activation of a diverse set of genes during the tobacco resistance response to TMV is independent of salicylic acid; induction of a subset is also ethylene independent. *Plant J* 21: 409–418

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* 227: 1229–1231

Khan RS, Nishihara M, Yamamura S, Nakamura I, Mii M (2006) Transgenic potatoes expressing wasabi defensin peptide confers partial resistanceto gray mold (*Botrytis cinerea*). *Plant Biotechnol* 23: 179–183

Kanzaki H, Nirasawa S, Saitoh H, Ito M, Nishihara M, Terauchi R, Nakamura I (2002) Overexpressing of wasabi defensin gene confers enhanced resistance to blast fungus (*Magnaporthe grisea*) in transgenic rice. *Theor Appl Genet* 105: 809–814

Kiba A, Saitoh H, Nishihara M, Omiya K, Yamamura S (2003) C-terminal domain of a hevein-like protein from *Wasabia japonica* has potent antimicrobial activity. *Plant Cell Physiol* 44: 296–303

Kiba A, Nishihara M, Tsukatani N, Nakatsuka T, Kato Y, Yamamura S (2005) A peroxiredoxin Q homolog from gentians is involved in both resistance against fungal disease and oxidative stress. *Plant Cell Physiol* 46: 1007–15

Kuc J (1972) Phytoalexins. *Ann Rev Phytopathol* 10: 207–232

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell* 79: 583–593

Linthorst HJM (1991) Pathogenesis-related proteins of plants. *Critic Rev Plant Sci* 10: 123–150

Mauch F, Staehelin A (1989) Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3 glucanase in bean leaves. *Plant Cell* 1: 447–457

Mishiba K, Nishihara M, Nakatsuka T, Abe Y, Hirano H, Yokoi T, Kikuchi A, Yamamura S (2005) Consistent transcriptional silencing of 35S-driven transgenes in gentian. *Plant J* 44: 541–556

Niderman T, Bruyere T, Gees R, Stintzi A, Legrand M, Fritig B, Mosinger E (1995) Pathogenesis-related PR-1 are antifungal. *Plant Physiol* 108: 17–27

Niki T, Mitsuhara I, Seo S, Ohtsubo N, Ohashi Y (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol* 39: 500–507

Ohashi Y, Ohshima M (1992) Stress-induced expression of gene for pathogenesis-related proteins in plants. *Plant Cell Physiol* 33: 819–826

Ono H, Tesaki S, Tanabe S, Watanabe M (1998) 6-methylsulfinylhexyl isothiocyanate and its homologues as food-originated compounds with antibacterial activity against *Escherichia coli* and *Staphylococcus aureus.* *Biosci Biotechnol Biochem* 62: 363–365

Osborn RW, De Samblanx GW, Thevissen K, Goderis I, Torrekens S, Van Leuven F, Attenborough S, Rees SB, Broekaert WF (1995) Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae. *FEBS Lett* 368: 257–262

Pedras MSC, Sorensen JL, Okanga FI, Zaharia I (1999) Wasalexins A and B, new phytoalexins from wasabi: isolation and synthesis, and antifungal activity. *Bioorgan Medic Chem Lett* 9: 3015–3020

Raikhel NV, Lee H-I (1993) Structure and function of chitin-binding proteins. *Annu Rev Plant Physiol Plant Mol Biol* 44: 591–615

Rauscher M, Adam AL, Wirtsz S, Guggenheim R, Mendgen K, Deising HB (1999) PR-1 protein inhibits the differentiation of rust infection hyphae in leaves of acquired resistant broad bean. *Plant J* 19: 625–633

Saitoh H, Kiba A, Nishihara M, Yamamura S, Suzuki K, Terauchi

R (2001) Production of antimicrobial defension in *Nicotiana benthamiana* with a potato virus X vector. *Mol Plant Microbe Interact* 14: 111–115

Sjahril R, Chin DP, Khan RS, Yamamura S, Nakamura I, Amemiya Y, Mii M (2006) Transgenic *Phalaenopsis* plants with resistance to *Erwinia carotovora* produced by introducing wasabi defensin gene using *Agrobacterium* method. *Plant Biotechnol* 23: 191–194

Thomma BP, Cammue BP, Thevissen K (2002) Plant Defensins. *Planta* 216: 193–202

Tomiyama K (1967) Further observation on the time requirement for hypersensitive cell death of potatoes infected by *Phytophthora infestans* and its relation to metabolic activity. *Phytopathologische Zeitschrift* 58: 367–373

Vance CP, Kink IK, Sherwood RT (1980) Lignification as a mechanism of disease resistance. *Ann Rev Phytopathol* 18: 259–288

van Loon LC, van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol Mol Plant Pathol* 55: 85–97