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Original Paper

Transgenic potatoes expressing wasabi defensin peptide confer partial resistance to gray mold (*Botrytis cinerea*)

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Abstract Five potato (*Solanum tuberosum* L.) cultivars were transformed with *Agrobacterium tumefaciens* strain EHA101 harboring wasabi defensin gene (isolated from *Wasabia japonica* L.) in a binary plasmid vector, pEKH1. The infected tuber explants co-cultivated for 3 days resulted in higher transformation efficiency (7–50% higher) for all cultivars than 2 days co-cultivation. PCR analysis showed an amplified fragment of wasabi defensin gene and the selectable marker, *nptII* gene in the genomic DNA of all clones rooted on MS medium supplemented with 100 $mg l^{-1}$ kanamycin, suggesting their transgenic nature. Southern blot analysis confirmed that transgenic plants integrated 1–6 copies of wasabi defensin gene into their genome. Expression of wasabi defensin protein was confirmed in the leaf extracts of independent transgenic clones by Western blot analysis. Antifungal assay of detached leaves from non-transformed control and transgenic plants indicated that transgenic plants were partially resistant to the fungal pathogen, *Botrytis cinerea* (gray mold).

Key words: *Botrytis cinerea*, *Solanum tuberosum*, transformation, wasabi defensin.

Phytopathogens are responsible for considerable losses ($30–$50 billion annually) in cultivated and stored crops (Baker et al. 1997). Applications of pesticides as countervailing approaches not only significantly increase production costs and are regarded as serious environmental hazards, but also contribute to the increase in antimicrobial-resistant species. The potato is the fourth most important food crop after wheat, maize and rice in world production for human consumption (Ross 1986). Various fungal and bacterial pathogens cause considerable losses in potato (Walter et al. 2001). Expression of antimicrobial peptides (AMPs) in plants seems to be a promising approach to confer broad-spectrum resistance to phytopathogens (Bradshaw and Mackay 1994; Broekaert et al. 1995, 1997; Cammue et al. 1992, 1994). In potato, a few studies have been reported on the integration and expression of antimicrobial proteins. Gao et al. (2000) demonstrated that the alfalafa antifungal peptide (alfAFP) defensin, isolated from seeds of *Medicago sativa*, showed strong activity against the fungal pathogen, *Verticillium dahliae*, in potato under greenhouse and field conditions. Transgenic potato with the gene encoding antimicrobial cationic peptide, temporin A was reported to be resistant to late blight (*Phytophthora infestans*) and pink rot (*Phytophthora erythroseptica*) (Osusky et al. 2004). However, AMPs from other plant sources have not been investigated in potato for possible broad-spectrum antimicrobial activity.

Wasabi (*Wasabia japonica* L.), a Japanese horseradish, has been a potential source of antimicrobial proteins. Antimicrobial protein, WjAMP-1 gene, isolated from leaves of *Wasabia japonica*, inhibited fungal and bacterial growth when expressed in *Nicotiana benthamiana* (Saito et al. 2001; Kiba et al. 2003). Transgenic tobacco plants overexpressing wasabi defensin gene were also shown to have antifungal activity against *Botrytis cinerea* (Nishihara, unpublished). Kanzaki et al. (2002) have reported that growth of blast fungus was inhibited in transgenic rice overexpressing wasabi defensin gene. These results suggest that the broad-spectrum antimicrobial activity of wasabi defensin might be effective for potato, which is susceptible to a number of fungal, bacterial and other pathogens. In the present study, therefore, integration and expression of wasabi defensin gene in potato via

Abbreviations: AMPs, antimicrobial peptides; DIG, Digoxygenine; GA, Gibberellic acid; HRP, horseradish peroxidase; IAA, indole-3-acetic acid, KDa, kilo Dalton; MM, modified MS medium; MS, Murashige and Skoog; nptII, neomycin phosphotransferase II; PVDF, polyvinylidine difluoride; SDS, sodium dodecyl sulphate; WjAMP-1, Wasabia japonica antimicrobial protein-1
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Agrobacterium tumefaciens-mediated transformation is described. Furthermore, antifungal activity of the transgenic plants thus obtained was evaluated by infecting with *Botrytis cinerea* as a model system.

A. tumefaciens strain EHA101 which harbors the binary vector, pEKHWT that contains the chimeric defensin gene (approximately 0.5 kb), isolated from *Wasabia japonica* (Saito et al. 2001; Kiba et al. 2003), and kanamycin and hygromycin resistance genes, was constructed for the present study and used (Figure 1A).

Tubers of 5 Japanese commercial potato cultivars, Waseshiro, Benimaru, May Queen, Irish Cobbler and Nishiyutaka were used for transformation. Inoculum preparation and transformation were carried out as described by Ishige et al. (1991) with slight modification. Briefly, tuber discs, 1–2 mm thick and 5×5 mm, were infected with the *Agrobacterium suspension* (diluted with hormone-free MS medium to $A_{600}=0.5$) for 6–8 minutes, blotted dry with sterilized filter paper to remove excess bacteria, and co-cultivated on $8\,g\,l^{-1}$ agar (Wako pure chemical industries, Japan)-solidified modified MS medium (MM), which consisted of MS salts and Nitsch vitamins supplemented with $20\,g\,l^{-1}$ sucrose, $0.2\,mg\,l^{-1}$ IAA, $2\,mg\,l^{-1}$ zeatin and $0.02\,\mu g\,l^{-1}$ acetosyringone, for 2 and 3 days under continuous dim light condition with cool white fluorescent lamps (Figure 2A-a). Controls were treated in the same way but without *Agrobacterium* infection. All the explants of a cultivar were prepared from a single tuber, and 25 explants were used per treatment.

After co-cultivation, the explants were washed with liquid hormone-free MM containing $10\,mg\,l^{-1}$ meropenem (Sumitomo Pharmaceuticals, Osaka, Japan) and transferred on to $8\,g\,l^{-1}$agar-solidified MM containing $20\,g\,l^{-1}$ sucrose, $0.2\,mg\,l^{-1}$ IAA, $2\,mg\,l^{-1}$ zeatin, $50\,mg\,l^{-1}$ kanamycin as selective chemical and $20\,mg\,l^{-1}$ meropenem as bactericide. One week after infection, the explants turned green, and small greenish nodular calli appeared on the upper surface of the explants (Figure 2A-b). After 2 weeks, the explants were transferred to $8\,g\,l^{-1}$agar-solidified MM supplemented with $20\,g\,l^{-1}$ sucrose, $2\,mg\,l^{-1}$ zeatin, $2\,mg\,l^{-1}$ GA3, $100\,mg\,l^{-1}$ kanamycin and $20\,mg\,l^{-1}$ meropenem for shoot formation. The nodular calli regenerated into shoots on this medium 4–6 weeks after co-cultivation. Approximately 2 months after co-cultivation, 2–3 adventitious shoots of 1–2 cm long (Figure 2A-c) were excised from the discs and cultured on $3\,g\,l^{-1}$ gellan gum (Gelrite; Kelco, Division of Merck, San Diego, CA)-solidified MM supplemented with $100\,mg\,l^{-1}$ kanamycin and $15\,mg\,l^{-1}$ meropenem for rooting. Putative transgenic shoots rooted within 1–2 weeks after culturing on this medium (Figure 2A-d), and finally only one well-rooted shoot was selected from each explant for further analysis, as an independent clone. These independent clones were cut into internodal segments and subcultured on the rooting medium for testing rooting ability and clonal propagation.

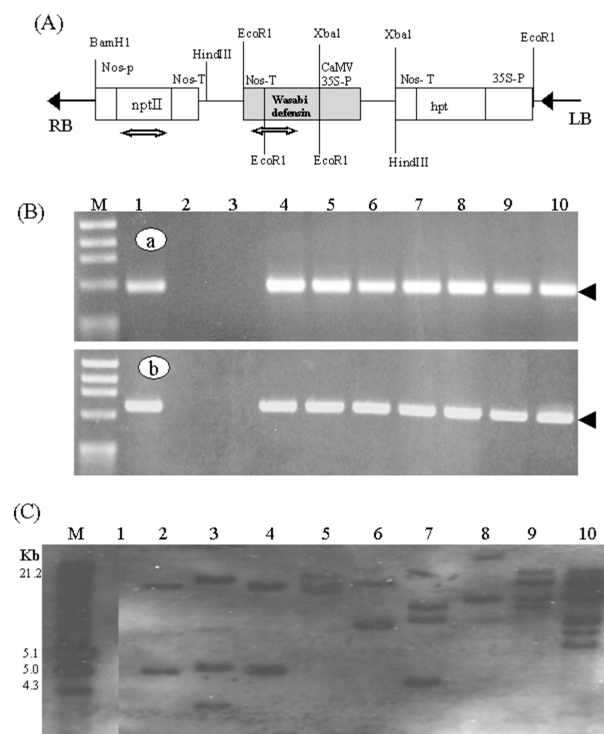

Figure 1. (A) T-DNA region of the binary vector, pEKHWT used for potato transformation. The genes for wasabi defensin and hygromycin phosphotransferase (*hpt*) are driven by CaMV 35S promoter, and the gene for neomycin phosphotransferase (*nptII*) by nopaline synthase promoter (*nos-p*). Double arrows, PCR-amplified regions used to confirm the existence of the defensin and *nptII* genes in transgenic plants. RB and LB, Right and left border sequences of the T-DNA region, respectively. CaMV 35S-P, cauliflower mosaic virus 35S promoter, nos-T, terminator of the nopaline synthase gene. Recognition sites of restriction enzymes are also indicated. (B) PCR analysis of putative transformed potato clones (cv. Waseshiro). (a) Amplification of wasabi defensin gene. (b) Amplification of *nptII* gene. Lane M, size marker ($\phi 174$/*Hae*III digests). Lane 1, positive control (plasmid DNA). Lane 2, non-transformed control. Lane 3, blank (without template). Lanes 4–10, independent transgenic potato plants. Arrows indicate the amplified fragments of wasabi defensin (0.5 kb) and *nptII* (0.8 kb) genes in transgenic plants. (C) Southern blot analysis of cv. Waseshiro. Genomic DNA from transgenic and non-transformed control potato plants was digested with *Xba*I, and hybridized with a DIG-labeled fragment containing the wasabi defensin gene as the probe. Lane M, DIG-labeled molecular weight marker III. Lane 1, non-transformed control. Lanes 2–10, independent transgenic clones. Transgenes copy number (1–6) in each transgenic clone is indicated by the bands with different locations.

In this study, we also analyzed the effect of co-cultivation period on transformation efficiency. There have been no studies on the effect of co-cultivation period as a factor affecting transformation efficiency. Although many researchers have used 2–4 days of co-cultivation periods for transformation of potato (Dale and Hampson 1995; Rockhold et al. 2001; Ishige et al. 1991), results of our transformation experiments,

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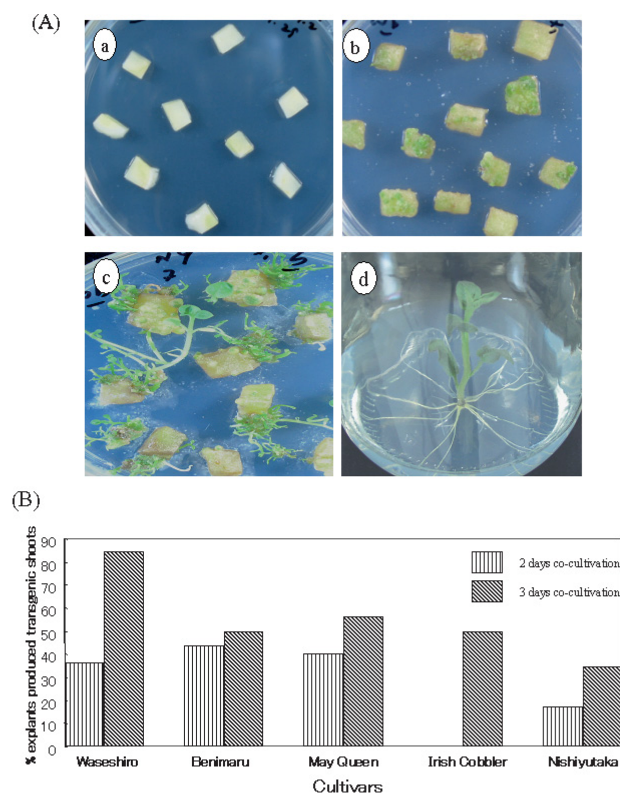

Figure 2. (A) Regeneration of potato tuber discs and production of transgenic plants after infection with *A. tumefaciens* harboring wasabi defensin gene. (a) Tuber discs on co-cultivation medium. (b) Nodule formation on discs before regeneration 3 weeks after co-cultivation. Note several regeneration events on a single tuber disc. (c) Shoot formation by tuber discs 6 weeks after co-cultivation. (d) A transgenic potato plant rooted on kanamycin containing MS medium. (B) Effect of co-cultivation period and cultivars on transformation efficiency of potato tuber discs infected with *A. tumefaciens* harboring wasabi defensin gene. After infection with the *A. tumefaciens* (diluted to $A_{600}=0.5$), the explants were co-cultivated for 2 and 3 days in continuous fluorescent dim light. Transformation efficiency is shown as % explants produced transgenic shoots. Co-cultivation for 3 days resulted in higher transformation efficiency than 2 days.

shown in Figure 2B, obviously indicated that co-cultivation for 3 days was better than 2 days for obtaining higher transformation efficiency for all cultivars tested. Especially in Irish Cobbler, 50% of transformation efficiency, i.e., percentage of explants that produced transgenic shoots, was obtained in 3 days of co-cultivation, whereas no transgenic plants were obtained in 2 days of co-cultivation. Transformation efficiency is also cultivar-dependent (Figure 2B), and Waseshiro, an important Japanese cultivar, showed the highest transformation efficiency, 84.6% with 3 days co-cultivation period, followed by May Queen (56.5%). Overall we obtained 20–40% higher transformation efficiencies than those previously reported using tuber dsics transformation (Sheerman and Bevan 1987; Ishige et al. 1991; Dale and Hampson 1995). This high transformation efficiency might be attributed to differences in co-cultivation periods, optimized transformation protocol and agronomic and physiological characteristics of the cultivars.

The results on PCR analysis performed using genomic DNA as a target and the oligonucleotide primers (Bex Co, Ltd Japan) for wasabi defensin and *nptII* genes indicated that all clones tested showed an amplified fragment of approximately 0.5 kb for wasabi defensin (Figure 1B-a) and 0.8 kb for *nptII* (Figure 1B-b) genes, suggesting that the T-DNA of the binary plasmid vector was successfully integrated into the genome of transgenic plants.

For Southern analysis, 10 μg of *Xba*I-digested DNA samples from transgenic and non-transgenic control plants were separated on a 0.8% (w/v) agarose gel, blotted to nylon membrane (hybond-N, Amersham Pharmacia Biotech) and hybridized with a DIG-labeled probe of the defensin gene. Hybridization, washing and detection were performed using DIG Easy Hyb (hybridization solution) and DIG Wash and Block Buffer set following the supplier's instructions (Boehringer Mannheim). The unique banding patterns (Figure 1C Lanes 2–10) indicate that the transgenic clones have originated from independent transformation events with 1–6 transgene integration sites. No transgene insertion was detected in non-transformed control (Figure 1C Lane 1).

To evaluate the expression of the integrated wasabi defensin gene in genomic DNA of transgenic plants, total proteins were extracted from 40 mg of fresh leaf tissues (leaf at position 3 or 4 from apex) from transgenic and non-transgenic control in vitro, 4–5 weeks old (containing 5–7 mature leaves) potato plants. After grinding the samples in liquid nitrogen, they were homogenized with extraction buffer [(62.5 mM Tris-HCl, pH 6.8, 2% (v/v) SDS (sodium dodecyl sulphate), 10% (v/v) glycerol)] and 0.2% β-mercaptoethanol. The homogenized samples were boiled for 3 min followed by incubation on ice for 2 min. Then the samples were centrifuged (20,000×g) for 5 min at 40°C. Total proteins in the supernatant from each sample were separated on 15% SDS-PAGE (Laemmli 1970) and electroblotted onto a polyvinylidine difluoride (PVDF) membrane (Amersham). Detection of the wasabi defensin protein was performed using polyclonal antisera raised in rabbit against the defensin protein (primary antibody, 1 : 1,000, v/v) and goat-anti-rabbit IgG (Amersham) conjugated to horseradish peroxidase (HRP) as secondary antibody (1 : 100,000, v/v).

The 5 KDa peptide of the wasabi defensin was detected as a single band by the wasabi defensin antibody in the total protein extracts from the transgenic plants with varying levels of expression in different clones (Figure 3A). The control plant did not express the defensin protein at all.

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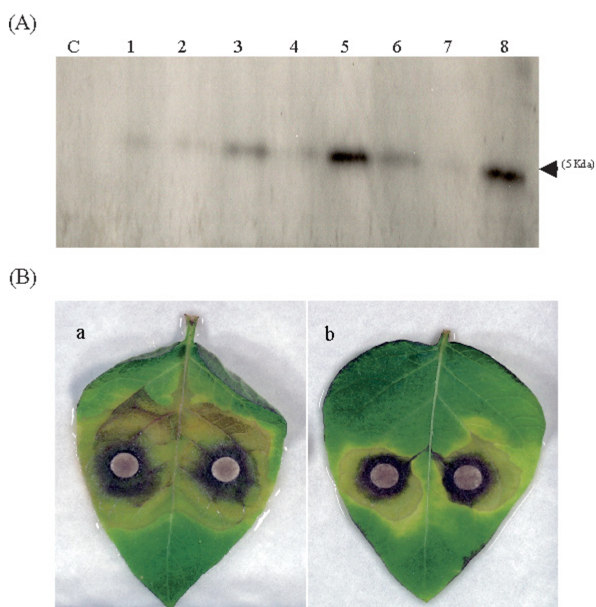

Figure 3. (A) SDS-PAGE analysis of the expression of wasabi defensin protein in *Solanum tuberosum* L. cv. Waseshiro. Protein extracts of leaves from in vitro grown transgenic independent clones and non-transformed control plant were resolved in a 15% polyacrylamide gel and subjected to immunoblot analysis using a rabbit polyclonal antiserum for wasabi defensin. C, non-transformed control. Lanes 1–8, transgenic independent clones. Arrow indicates the 5 KDa band of the wasabi defensin protein, note varying levels of expression in different clones. (B) Assay of resistance to the fungal pathogen, *Botrytis cinerea*, in transgenic potato (cv. Waseshiro). Detached leaves from 4 to 6-weeks old control (a) and transgenic (b) plants were infected with the spores of *B. cinerea* and incubated at 23°C, 16 h light/8 h dark conditions and high humidity. Pictures were taken 3 days after infection. Disease intensity was inferred from the lesion size around the inoculation spot. Wasabi defensin partially restricted growth of the fungus in transgenic plants (b).

We evaluated the antimicrobial activity of wasabi defensin against *Botrytis cinerea*, a phytogenic fungus which does not exhibit high host specificity. Under favorable climatic and physiological conditions, it is capable of growing on all species of dicotyledons. The disease is generally not economically important, but its importance in potato can be highlighted in two ways: firstly, other tuber rotting fungi and bacteria can invade gray mold lesions and cause a soft rot; secondly, gray mold can mask other disease symptoms and make accurate disease diagnosis difficult.

Botrytis cinerea (gray mold strain 40) was grown in YM culture medium ($10\,g\,l^{-1}$ glucose, $5\,g\,l^{-1}$ Polypeptone, $3\,g\,l^{-1}$ Malt Extract, $3\,g\,l^{-1}$ Yeast Extract) and spore concentration was adjusted to $1\times10^6\,m\,l^{-1}$. As an inoculation method, $50\,\mu l$ of spore solution was applied to a round filter paper (ϕ7 mm) on press-injured spots (2 mm in diameter) of leaves (2 spots per leaf) (Figure 3B) from 3 transgenic clones and non-transgenic control potato plants (cv. Waseshiro). After inoculation, the leaves were kept at 23°C under 16 h light/8 h dark conditions and high humidity for 3 days. Disease intensity was inferred from the lesion size developed from inoculated spots on the leaves. The results showed that there was obvious difference in the response between transgenic clone 3 (the clone of lane 5 in Figure 3A) and the control in terms of the lesion size around the inoculation spot (Figure 3B-a & b). The wasabi defensin peptide partially restricted growth of *B. cinerea* in the transgenic plant, verifying its antifungal activity. However, antifungal activity of the other two clones was not obviously different from that of non-transgenic control (data not shown).

These results revealed that the wasabi defensin gene was successfully integrated into the genome of transgenic potato plants and expressed by producing the defensin protein. Expression of the antimicrobial peptide, wasabi defensin, demonstrated partial resistance to *B. cinerea* in transgenic potato. However, further research is needed to produce transgenic plants of potato with more resistance to *B. cinerea* and to test the resistance to other pathogenic fungi and bacteria such as *Phytophthora infestans*, *Alternaria solani*, *Erwinia carotovora*, and *Streptomyces* spp.

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