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Short Communication

Transgenic *Phalaenopsis* plants with resistance to *Erwinia carotovora* produced by introducing wasabi defensin gene using *Agrobacterium* method

Rinaldi Sjahril[1,2], Dong Poh Chin[1], Raham Sher Khan[1], Saburo Yamamura[3], Ikuo Nakamura[1], Yoshimiki Amemiya[1], Masahiro Mii[1]*

[1] Faculty of Horticulture, Chiba University, 648 Matsudo, Matsudo City, Chiba 271-8510, Japan; [2] Faculty of Agriculture and Forestry, Hasanuddin University, Jl. Perintis Kemerdekaan Km 10, Makassar 90245, South Sulawesi, Indonesia; [3] Iwate Biotechnology Research Center, 22-174-4 Narita, Kitakami City, Iwate 024-0003, Japan
*E-mail: miim@faculty.chiba-u.jp Tel & Fax: +81-47-308-8852

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Abstract Transgenic plants over-expressing wasabi defensin gene were successfully produced in *Phalaenopsis* orchid by *Agrobacterium*-mediated transformation method. Embryogenic cell suspension culture of *Phalaenopsis* Wataboushi '#6.13' was infected with *A. tumefaciens* strain EHA101 carrying a plasmid containing wasabi defensin gene and selectable marker *npt*II, *hpt* genes. Plantlets were regenerated through somatic embryogenesis from the calli selected on hygromycin-containing medium. Transformation of plantlets with wasabi defensin gene was confirmed by PCR analysis. Southern blot analysis confirmed successful integration of 1–4 copies of the gene. Production of the 5 kDa wasabi defensin protein with varying levels was confirmed in the leaf extracts of different transgenic clones using Western blot analysis. Most of the transgenic plants showed strong resistance to *Erwinia carotovora*, which causes soft rot disease in the control plant. These results suggest the usefulness of this gene for conferring the resistance to various diseases of *Phalaenopsis* and possibly other orchids.

Key words: *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Phalaenopsis*, wasabi defensin.

Genetic improvement of *Phalaenopsis* for conferring disease resistance by conventional plant breeding (sexual hybridization) is restricted by the lack of available useful genes in the species of *Phalaenopsis* and related genera. Consequently, genetic transformation method is expected to apply for transferring disease resistance genes as an alternative procedure. So far, transformation with direct delivery of marker genes such as GUS and GFP genes into plant cells by particle bombardment and indirectly through the use of *Agrobacterium*-mediated procedure has been reported on orchids (Anzai et al. 1996; Belarmino and Mii 2000; Chia et al. 1994). However, there have been no reports on the transformation of useful genes including plant disease resistance genes into *Phalaenopsis*.

Plants react against pathogen attacks in a variety of defensive way such as hypersensitive cell death, production of phytoalexins and expression of pathogenesis-related proteins including a number of antimicrobial peptides (Kiba et al. 2003). So far, many antimicrobial proteins such as chitin-binding proteins, thionins, lipid-transfer proteins and defensins have been identified and their activity has been tested against fungi and bacteria (Florack and Stiekema 1994; Broekaert et al. 1995; Terras et al. 1995).

Plant defensin protein families have been detected in different tissues from wide range of plants such as wheat, barley, pepper, mustard and radish. They are cystein-rich polypeptides of 5 kDa in size and are considered to have defensive role against plant pathogenic bacteria (Bohlmann 1994) and fungi (Thevissen et al. 1996). Integration and over-expression studies of defensin gene has been reported in many transgenic dicotyledonous plants such as *Arabidopsis*, tobacco, potato, mung bean and canola (Eppel et al. 1997; Gao et al. 2000; Saitoh et al. 2001; Kiba et al. 2003) and only few studies were reported on monocotyledonous plants such as rice, wheat (Kanzaki et al. 2002; Liang et al. 1998) and banana (Chakrabarti et al. 2003).

The Japanese horseradish, wasabi (*Wasabia japonica*),

Abbreviations: GFP, green fluorescence protein; GUS, β-glucuronidase; NDM, New Dogashima medium; NDM-10M, New Dogashima medium with 1% maltose; NDM-20S, New Dogashima medium with 2% sucrose; PGR, plant growth regulator; PLB, protocorm-like body; PSA, potato sucrose agar; WjAMP-1, *Wasabia japonica* antimicrobial protein.
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which has a protective role against microorganisms and has been used as a food ingredient, are considered to have some antimicrobial substances. Secondary metabolites of wasabi, such as wasalexin and 6-methylsulfonylhexyl isothiocyanate, have been reported to have antifungal and antibacterial activity, respectively (Pedras et al. 1999; Ono et al. 1998). Recently, antimicrobial protein-encoding gene (WjAMP-1) was isolated from wasabi (Saitoh et al. 2001) and named as wasabi defensin gene by Kanzaki et al. (2002). Antimicrobial protein, coded by WjAMP-1 gene showed a strong expression and inhibitory effect on fungal and bacterial growth in transgenic *Nicotiana benthamiana* (Saitoh et al. 2001). Furthermore, Kanzaki et al. (2002) have reported that growth of rice blast fungus was inhibited in transgenic rice over-expressing the wasabi defensin gene.

In this study, we report an attempt to transfer wasabi defensin gene derived from a dicotyledonous plant, *Wasabia japonica* into monocotyledonous plant, *Phalaenopsis* orchid, and evaluate their resistance to bacterial pathogens.

Fine embryonic cell suspension culture of *Phalaenopsis* Wataboushi '#6.13', which was induced and maintained from meristem culture of axillary bud as described previously (Tokuhara and Mii 1993) was used for the transformation study. Five day-old suspension cells after each subculture were used for the transformation experiments.

The *A. tumefaciens* strain EHA101 (pEKH-WT) used harbored the wasabi defensin gene isolated from *Wasabia japonica* (Figure 1). Inoculation of the suspension cells with the *Agrobacterium* was performed following the method of Belarmino and Mii (2000) with some modifications (Sjahril and Mii in press), by using 2 h of inoculation instead of 10 h recommended. Also, instead of using 500 mg l^{-1} cefotaxime for bacteria elimination, cells were washed with 10 mg l^{-1} meropenem for 30 min and then transferred onto new sterilized filter paper placed on 40 ml selective medium, which is 2.5 g l^{-1} Gelrite-solidified NDM-20S supplemented with 25 mg l^{-1} hygromycin and 5 mg l^{-1} meropenem. Meropenem was omitted after the fifth or sixth subculture at 2 week-intervals by visually confirming the absence of *Agrobacterium*.

Most of the cells turned brown and died after 4 to 6 weeks on selection medium. After another month, small creamy white cell colonies with hygromycin-resistance initiated to grow among browned or dead cell population and formed small greenish yellow calli. Approximately 19 hygromycin-resistant callus clones were selected per 1 g of cells co-cultivated with *Agrobacterium* in three separate transformation experiments. The yield of hygromycin-resistant calli was much higher than that obtained in the previous report (Belarmino and Mii 2000), in which 1 g cell clumps or clusters only yielded about five hygromycin-resistant clones after selection with 50 mg l^{-1} hygromycin. The use of finer cell aggregates and lower concentration of hygromycin in the present study might have resulted in the

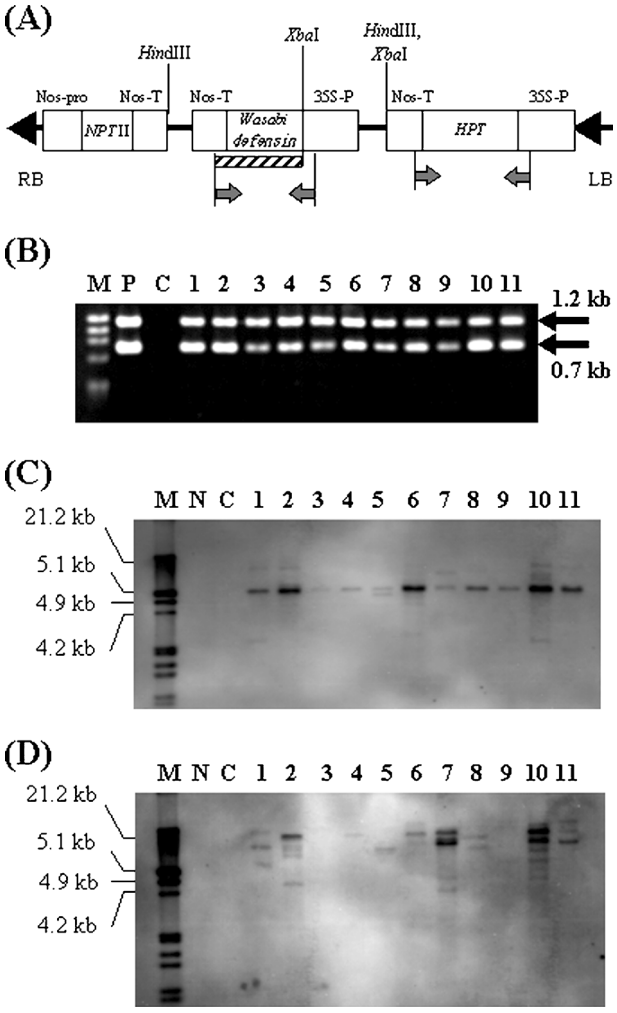

Figure 1. T-DNA region of the binary vector pEKH-WT used for *Phalaenopsis* genetic transformation and molecular analyses of the transgenic plants. (A) T-DNA regions of the binary vector pEKH-WT. The genes for wasabi defensin and hygromycin phosphotransferase (*hpt*) are driven by CaMV 35S promoter and the gene for neomycin phosphotransferase (*npt*II) is driven by nopaline synthase promoter (*nos-pro*). *RB* and *LB* Right and left border sequences of the T-DNA region, respectively. *Bold arrow*, PCR-amplification regions for confirming *hpt* gene (1.2 kb) and wasabi defensin gene (0.7 kb). *Diagonal lined box* Regions used as the probe for wasabi defensin gene. *CaMV 35S-P* Cauliflower mosaic virus 35S promoter. *Wasabi defensin* Wasabi defensin cDNA, *Nos-T* Terminator of the nopaline synthase gene. *Hin*dIII, *Xba*I Restriction enzyme recognition sites. (B) Detection of *hpt* (1.2 kb) and wasabi defensin (0.7 kb) genes in the putative transgenic *Phalaenopsis* plantlets by PCR. *Lane M* molecular size marker (*ϕX*174/*Hae*III), *lane P* plasmid pEKH-WT as positive control. *Lane C* non-transformed control, *lanes 1–11* different transgenic *Phalaenopsis* plantlets #1 to #11. (C) and (D) Southern blot analysis of genomic DNA of transgenic plantlets, digested with *Hin*dIII (C) and *Xba*I (D), respectively. *Lane C* non-transformed control plant, *lanes 1–11* transgenic plantlets. *Lane M* digoxigenin (DIG)-labeled molecular marker, *lane N* negative control.

higher yield probably because of more chance to make *Agrobacterium* cells contact directly with the *Phalaenopsis* cells. The fine cell aggregates after infection might also be exposed directly to the selection antibiotics, resulting in less escaped calli.

Putative transgenic callus selected were transferred onto 2.5 g l^{-1} Gelrite-solidified NDM-20S containing 25 mg l^{-1} hygromycin for further proliferation. One month after the transfer, the calli were sub-cultured to PGR free Gelrite-solidified NDM containing 10 g l^{-1} maltose (NDM-10M) and the same hygromycin concentration for 1 month for callus greening. Green calli produced were subcultured onto NDM-10S containing 10 mg l^{-1} sucrose with 30 mg l^{-1} hygromycin for regeneration of PLBs and plantlets for 3–4 months with monthly subcultures.

Approximately 78% of the hygromycin-resistant callus clones, regenerated into hygromycin-resistant PLBs on PGR-free NDM-10S containing 30 mg l^{-1} hygromycin and about 30 transgenic plantlets were obtained from 1 g fresh weight of embryonic suspension calli, after 7–8 months of culture after infection.

The presence and integration of wasabi defensin gene in the plant genome was indicated by PCR of the extracted genomic DNA from putative transgenic plantlets. Total genomic DNAs were isolated from young leaves of putative transgenic plants and control plant following the CTAB method (Murray and Thompson, 1980). PCR reaction was performed using genomic DNA as a target and oligonucleotide primers (Bex Co. Ltd., Tokyo, Japan) for the wasabi defensin and *hpt* genes. All eleven hygromycin-resistant plantlets showed positive specific amplification of approximately 0.7 kb fragment for wasabi defensin gene (Figure 1B) and approximately 1.2 kb for *hpt* gene, respectively (Figure 1B), suggesting the transgenic nature of the *Phalaenopsis* plantlets analysed.

Integration and the number of insertion sites of the transgenes in the *Phalaenopsis* genome were examined by Southern blot analysis. Genomic DNA samples (10 μg) from hygromycin-resistant transgenic plantlets and control plantlet were digested with *Hin*dIII and *Xba*I and electrophoresed on a 0.8% (w/v) agarose gel, blotted to nylon membrane (Immobilon-NY$^+$, Millipore, Bedford, MA, USA) and hybridised with digoxigenin-labeled probe of the wasabi defensin gene following the manufacturer's instruction (Roche Diagnostics GmbH, Mannheim, Germany). The insertion of the wasabi defensin gene into the genome was confirmed in both the digest with *Hin*dIII (Figure 1C) and *Xba*I (Figure 1D), respectively, whereas it was not detected in non-transformed plant. Digestion with *Xba*I also revealed that the number of inserted T-DNA copies varied from 1 to 4 in the independent 11 transgenic clones (Figure 1D).

For Western blot analysis, total proteins were extracted

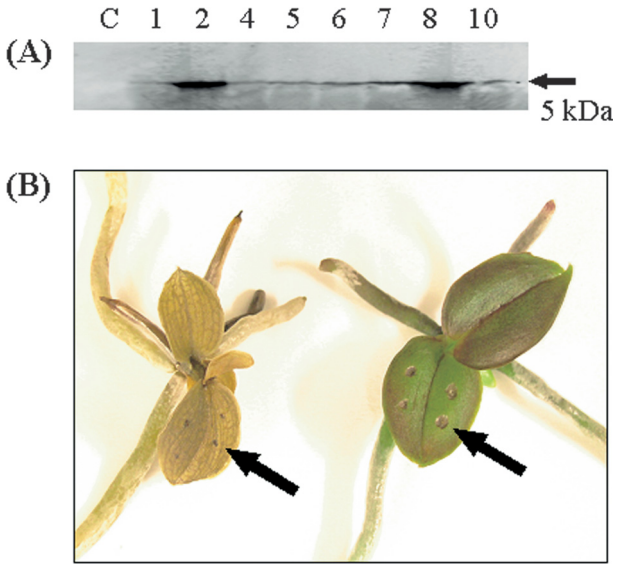

Figure 2. Defensin production and resistance of transgenic *Phalaenopsis* plant against *Erwinia carotovora*. (A) Western blot analysis of the expression of wasabi defensin protein in leaves of *Phalaenopsis* plantlets. *Arrow* indicates the 5 kD band of the wasabi defensin protein. Note the varying level of expression among different clones. C non-transformed control plantlet, *lanes 1*, *2*, *4*, *5*, *6*, *7*, *8*, *10* correspond to the different transgenic clone numbers shown in Figure 1B to 1D. (B) Evaluation of transgenic clone #2 of *Phalaenopsis* Wataboushi '#6.13' for resistance to bacterial soft rot, one week after the inoculation. *Black arrows* represent the points of inoculation with the bacteria. Note the soft rot symptom in the control plant (left), whereas no sign of symptom in the transformant (right).

from 50 mg of leaf tissues from non-transgenic and transgenic plantlets, fractionated by 15% SDS-PAGE and electro blotted onto PVDF membrane (Bio-Rad, Bio-Active Co. Hercules, CA, USA). Detection of the wasabi defensin protein was performed using ECL Advance Western Blotting Detection Kit (Amersham Bioscience, Buckinghamshire, UK). Antibodies used were polyclonal antisera raised in rabbit against the defensin protein (primary antibody at 1 : 500 v/v) and goat-anti-rabbit IgG conjugated to horseradish peroxide (HRP) as secondary antibody at 1 : 100,000 v/v.

As a result, the 5 kDa peptide which corresponds to the translated form of the wasabi defensin (Kanzaki et al. 2002), was detected as a single band in the leaves of transgenic clones but not in the non-transgenic control plant. The level of wasabi defensin expression varied among the clones (Figure 2A) and clones #2 and #8 showed relatively stronger expression. Similar difference in the expression levels of the same protein has also been reported in other species (Fagard and Vaucheret 2000; Kanzaki et al. 2002). It is interesting to note that the control plant showed no expression of the defensin protein at all, suggesting that *Phalaenopsis* might not have this specific anti-fungal protein at all or maybe very little.

In order to evaluate disease resistance of the

transgenic plant, bacterial strain of *Erwinia carotovora* was isolated from a *Phalaenopsis* plant severely showing a symptom of soft rot disease in a greenhouse and grown on potato sucrose agar (PSA; 500 ml of potato broth made from 200 g potato, 20 g sucrose and 15 g agar in 1000 ml Milli-Q water, pH 5.6). For testing resistance of transformant against *E. carotovora*, non-transformed and transgenic plantlets grown on Gelrite-solidified NDM-10S were infected with the bacteria by inoculating at 4 points of one leaf for each plant using sterile needles. One week after the infection treatment, all non-transformant plantlets tested were susceptible to *E. carotovora* and died with soft rot symptom, whereas transformed plantlets showed strong resistance against inoculation of the bacteria (Figure 2B). Out of 15 transgenic clones tested, almost all showed no symptom 1 week after inoculation except for 1 clone (6.7%), which showed some susceptibility and died 4 weeks after the treatment.

We have succeeded in efficiently producing transgenic plants with wasabi defensin gene of *Phalaenopsis* orchid by using the transformation protocol used in the present study. Since almost all the transgenic plants successfully showed strong resistance to *E. carotovora*, they are expected to be resistant to some other important diseases of this orchid.

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