An Evaluation of Disease Resistance of *Agrobacterium*-mediated Transgenic Potato (*Solanum tuberosum* L.) Containing the Chicken Lysozyme Gene or the Wild Spinach Chitinase Gene

Dai HIRAI[1], Takako SUZUKI[1], Daisuke YANAGIDA[2], Jun SASAKI[1], Toshimi KUSUME[3], Tadahiko KIGUCHI[1], Toru TAKEUCHI[1] and Hideto TAMAGAKE[1]

Agrobacterium-mediated genetic transformation of *Solanum tuberosum* L. was studied by introducing the chicken lysozyme gene and the wild spinach chitinase gene. Following co-incubation with *Agrobacterium* on modified White medium supplemented with 0.1mg/l IAA, GA_3 and 0.5mg/l zeatin for 2 days, tuber discs were transferred to the medium containing 200mg/l carbenicillin and 100mg/l kanamycin. Apical nodal segments of regenerated shoots were transferred to half strength MS medium containing 100mg/l kanamycin. Microtubers obtained from rooted plantlets which were certified the presence and the expression of transgenes were harvested and sowed in the isolated green house to obtain their tubers for disease resistance assays. One line designated EC100 was selected as resistant line against Dry Rot (*Fusarium avenaceum*) from transgenic lines harboring the wild spinach chitinase gene. R93 was also selected as resistant line against Black Leg (*Erwinia chrysanthemi*) from transgenic lines containing the chicken lysozyme gene. No differences were observed in characters of their appearance between transgenic lines and their non-transgenic cultivars.

Keywords: transformation, disease resistance, *Solanum tuberosum* L., the chicken lysozyme gene, the wild spinach chitinase gene, Dry Rot, Black Leg

Abbreviations: GA_3, Gibberellic acid; GUS, β-glucuronidase; IAA, Indole acetic acid; HEWL, Hen egg white lysozyme; MS medium, Murashige and Skoog medium[7]

Introduction

Potato is one of the most important field crops not only in Hokkaido but also in the world. In Hokkaido area, breeding programs and research for its cultivation and pest control have been held by Hokkaido prefectural agricultural experiment stations, national institutes and universities. Despite those efforts, decreasing grade quality and yield caused by diseases still remains as serious problems.

One of the priority purposes of potato breeding is developing new lines which are resistant to pests (fungal and bacterial diseases, virus, nematodes and insects). Recently, Kitami Agricultural Experimental Station released a new cultivar which was relative resistant to Common Scab. This disease caused by *Streptmyces scabies* (Thaxt.) Waksman & Henrici, produces tuber lesions which make disfigurement of potato tubers, yield loss, decreasing grade quality and storing ability. Kitami Agricultural Experiment Station has been releasing other lines and cultivars resistant to Late Bright caused by *Phytophthora infestans* (Montagne) de Bary and Potato Cyst Nematodes. They have been making efforts to select the resistant lines or cultivars to other pests from their genetic resources including wild relatives of cultivated potatoes, which are

Received 29, January, 2004

1 Hokkaido Central Agricultural Experiment Station, Naganuma, Hokkaido, 069-1395, Japan
E-mail:hiraidai@agri.pref.hokkaido.jp

2 ibid. (Present; Hokkaido Kitami Agricultural Experimental Station, Kunnneppu, Hokkaido, 099-1496 Japan)

3 ibid. (Present; Hokkaido Kamikawa Agricultural Experimental Station, Pippu, Hokkaido, 078-0397, Japan)

supposed to have many valuable genes conferring pest resistance. However, developing new pest resistant cultivars is still difficult and takes a long time because of genetic characters of potato, i.e. its ploidy and self-incompatibility[14]. In 1995, 32 diseases caused by fungi, bacteria and virus were reported to occur to potato in Hokkaido area. Thus, almost all of these diseases should be controlled by pesticides, modifications of cultivation system and well-controlled propagation of seed tubers. But some of them caused by soil borne pathogens, i.e. fungi and bacteria, are difficult to control by pesticides because they are in soil and directly attack the underground tubers. Common Scab, Dry Rot and Black Leg are well-known as diseases caused by soil borne pathogens.

Developing genetic engineering techniques gives opportunities for making disease resistant cultivars by transformation of foreign genes into potato plants. This technique is especially important when there are no desirable genes in the genetic resources. Potato is one of plants easy to be transformed and many transgenic cultivars were reported[11] and some of them have been released for commercial use since 1995[13]. As foreign genes for making transgenic potatoes which were resistant to virus, fungal and bacterial diseases, gene of coat proteins of the virus, anionic peroxidase gene and lysozyme gene and so on have been used[14].

The chitinase gene and the lysozyme gene are well-known as disease resistant genes. Both enzymes can degrade cell walls of fungi and/or bacteria in some degrees and suppress their growth. Many transgenic plants were reported to express these enzymes in the host plants and to increase or to add the resistances against some diseases to the host plants. In this study, the wild spinach chitinase gene and the chicken lysozyme gene were successfully inserted using *Agrobacterium* and those transformed potato lines expressed resistances against Dry Rot (the wild spinach chitinase gene), one of fungal diseases or Black Leg (the chicken lysozyme gene), one of bacterial diseases. These resistances were still experimental because degrees of their resistance to each disease were evaluated in our laboratory and the isolated green house. However, we demonstrated here that transgenic potatoes harboring foreign genes could increase or gain the pest resistances and show the same external appearance as their non-transgenic cultivars.

Materials and methods

Plant materials

Danshakuimo and May-queen were used in this study. Both cultivars were supplied by Kitami Agricultural Experiment Station as tubers and they were stored at 4℃ in the dark until use.

Agrobacterium solution

Agrobacterium tumefaciens (*Rhizobium radiobacter*[10]) strain LBA4404 containing pB2113-CAP9 plasmid or pBI121-L23Lys plasmid was used. pB2113-CAP9 plasmid had the wild spinach chitinase gene under regulation of El2Ω promoter, which were kindly supplied by Miyagi Agricultural Center and National Institute of Agrobiological Sciences, respectively. Plasmid pBI121-L23Lys had CaMV35S promoter and the chicken lysozyme gene, which was one of cDNAs isolated from oviduct of 'White leghorn' and its sequence was highly homologous to that of other chicken lysozyme (HEWL[12]) reported before. Same *Agrobacterium* strain containing pBI121-GUS plasmid was also used. All plasmids also contained the nos-nptII gene conferring Neomycin resistance to transgenic plants. DNA isolation from potato, southern hybridization, western hybridization, PCR were carried out according to the standard protocols[9]. *Agrobacterium* was grown in 5ml of YEP liquid medium at 25℃ for 16h. It was centrifuged and resuspended in MS[7] liquid medium until the absorbance (600nm) adjusted to 0.5.

Transformation and selection

Tubers were washed and cylinders (20mm in diameter) were punched out using a cork bore. They were surface sterilized by 0.25% sodium hypochlorite for 15min followed by rinsed 3 times with sterilized distilled water. Cylinders 10mm in diameter were punched out from them and cut into discs of approximately 1mm thickness. Discs were soaked in *Agrobacterium* solution for 10min and then placed on regeneration medium, which was modified White medium supplemented with 0.1mg/l IAA, GA_3 and 0.5mg/l zeatin[5] for 2 days at 25℃ in 16h photoperiod (co-incubation). These infected discs were transferred to selection medium which was the same medium supplemented with 200mg/l carbenicillin and 100mg/l kanamycin for 2 weeks intervals until shoots emerged from them.

Apical nodal segments were cut off from these shoots and transferred to root induction medium containing half strength of MS medium solidified with 7g/l agar and 100mg/l kanamycin. Rooted plantlets were selected as kanamycin resistant plantlets and then transferred to microtuber medium containing MS medium supplemented with 8% sucrose and 7g/l agar and incubated at 25℃ in dark for 4 to 6 weeks. At the same time, the presence of transgene and its expression of each kanamycin resistant plant were checked by PCR, southern hybridization and western hybridization. Microtubers of plants which were certified to be transformed were harvested and stored at 4℃ in dark for 4 to 8 weeks and then sowed in the isolated green house to obtain their tubers for subsequent experiments.

Assays of disease resistance

Tubers of transgenic plants were harvested from individual plants and then surface-sterilized as described before and sliced approximately 2mm thickness. These discs were placed on sterilized and moist filter paper in plastic dishes. A disc of each tuber was treated in each of 3 replicates. Tubers of selected lines were sowed in the isolated green house again to obtain their larger tubers to be assayed.

Fusarium avenaceum strain AG9 was cultured on potato-broth agar medium at 25℃ for 7 days. Discs of hyphae on the medium (5mm diameter) were punched out using cork bore. One hyphae disc was placed on a tuber disc and incubated at 25℃ in dark for 7 days. *Erwinia chrysanthemi* was cultured overnight at 25℃ in NB liquid medium on a rotary shaker (150rpm) and diluted by sterile distilled water to 10^7 cfu/ml. An aliquot of 0.01ml of bacteria suspension was placed on a tuber disc and incubated at 25℃ for 2 days in dark.

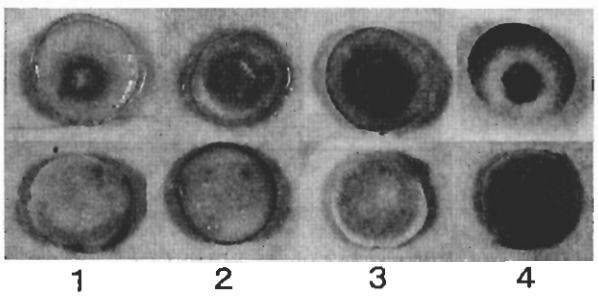

Figure 1 Disease index for Dry Rot (*Fusarium avenaceum*, upper) and Black Leg (*Erwinia chrysanthemi*, bottom)

Resistances against *Fusarium avenaceum* or *Erwinia chrysanthemi* of transgenic plants containing the wild spinach chitinase gene or the chicken lysozyme gene were qualified by disease index in tuber discs ranging from 0 to 4 (Figure 1). Disease indexes obtained from these assay were analyzed by Duncan's multiple range test for data comparison.

Result

Transformation

3058 tuber discs of May-queen and 373 tuber discs of Danshakuimo were co-incubated with A. *tumefaciens* carrying pB2113-CAP9 (the wild spinach chitinase gene) and pBI121-L23Lys (the chicken lysozyme gene) respectively. They were screened for regeneration ability on selection medium. 231 plantlets were emerged from infected discs, 93 plantlets of them rooted on agar medium containing kanamycin and 23 plantlets of them were confirmed the presence and the expression of transgene by PCR, southern hybridization and western hybridization (Table 1, 2). These transgenic plantlets successfully produced microtubers which grew and produced small tubers in the isolated green house after storage.

Table 1 Transformation frequency during different steps using the wild spinach chitinase gene. Cultivar: May-queen.

Procedures	Number of discs or plantlets	Frequency (%)
Tuber discs	3058	
Plantlets regenerated	199	6.5
Plantlets with kanamycin-resistance	81	2.6
PCR-positive plantlets	14	0.5
Plantlets produced microtubers	14	0.5
Dry rot-resistant plantlets	1	0.03

Table 2 Transformation frequency during different steps using the chicken lysozyme gene. Cultivar: Danshakuimo.

Procedures	Number of discs or plantlets	Frequency (%)
Tuber discs	373	
Plantlets regenerated	32	8.6
Plantlets with kanamycin-resistance	12	3.2
PCR-positive plantlets	9	2.4
Plantlets produced microtubers	9	2.4
Black leg-resistant plantlets	1	0.3

Assays of disease resistance

In the preliminary experiments, inoculation conditions were determined. 10^6, $5x10^6$, 10^7 and 10^8 of the number of colony formation units (Black Leg) and storage periods of tubers after harvest (0, 10, 30 days) (Black Leg and Dry Rot) were compared and the conditions mentioned in materials and methods were chosen as the optimal conditions for the selection of disease resistant plants.

Dry Rot: Microtubers of 14 transgenic plantlets (Table 1) produced small tubers in the isolated green house. 3 small tubers from each line were assayed and a line expressing a significantly lower disease index was selected (EC100, Figure 2). Tubers of this selected line sowed again and larger tubers were harvested. They showed the same tendency in disease index of Dry Rot (Table 3, Figure 3).

Blaek Leg: Microtubers of 9 transgenic plantlets (Table 2) produced small tnbers in the isolated green house and 3 small tubers from each line were assayed. R93

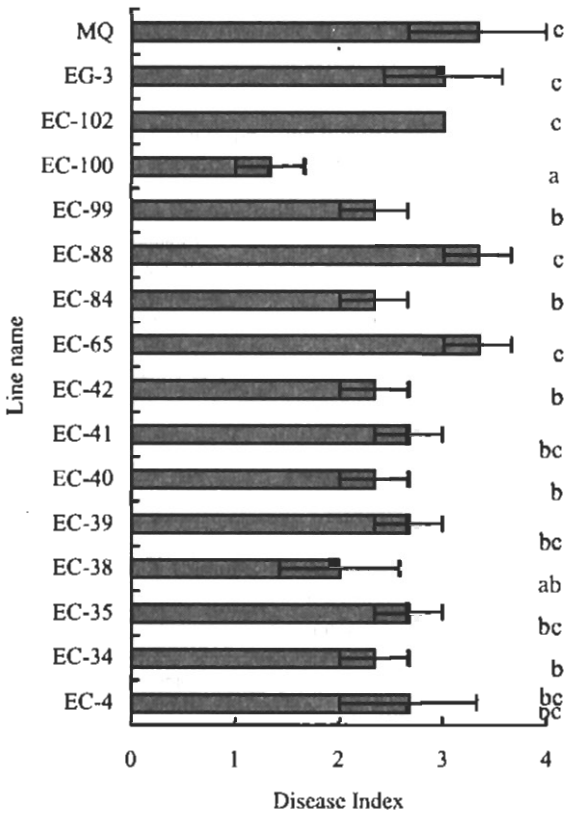

Figure 2 Disease index for Dry Rot of transgenic potato lines. Bars indicate standard errors. Data followed by the same letter are not different significantly at 5% level.

Table 3 Disease indexes (Dry Rot) of transgenic plant lines and non-transgenic cultivars.

Line	Disease index (Average ± SE)
EC100	1.17 ± 0.17 a
May-queen	3.66 ± 0.33 c
Danshakuimo	3.50 ± 0.29 c
EG3	2.67 ± 0.33 b

EG3: transgenic plant line using GUS gene (cultivar May-queen). SE stands for standard errors. Data followed by the same letter are not different significantly at 5% level.

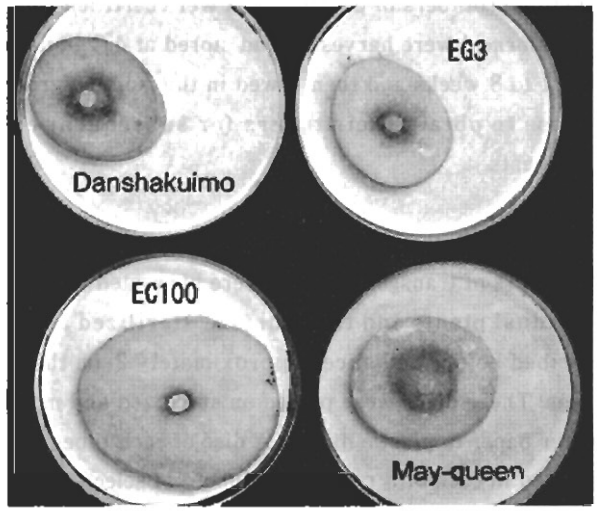

Figure 3 hyphae growth of *Fusarium avenaceum* (Dry Rot) on the tuber discs. Tuber discs of each line were inoculated and incubated for 7 days.

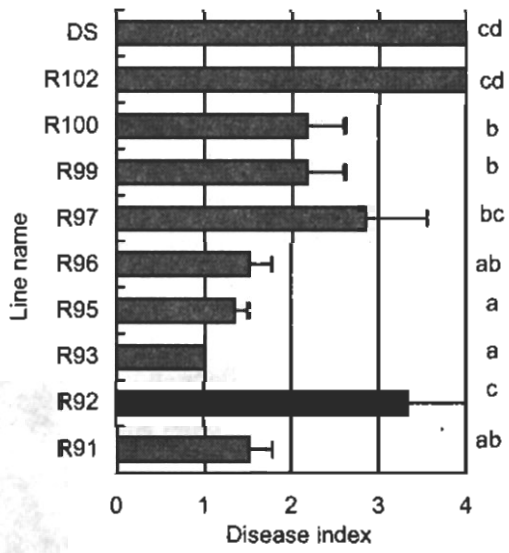

Figure 4 Disease index for Black Leg of transgenic potato lines. Bars indicate standard errors. Data followed by the same letter are not different significantly at 5% level.

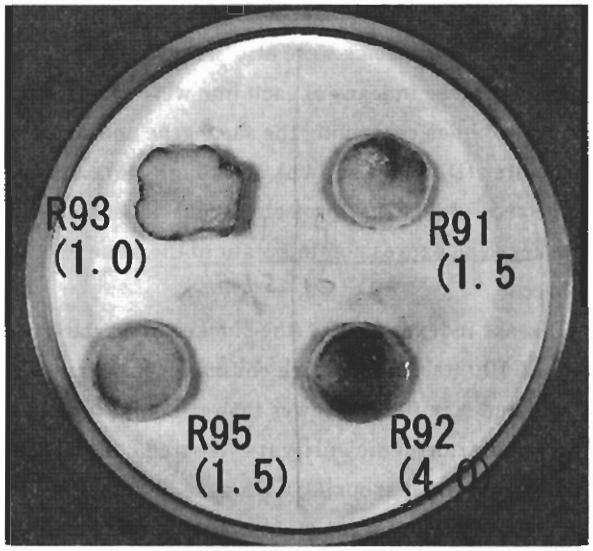

Figure 5 Disease indexes (in parentheses) of Black Leg. Tuber discs of each line containing the chicken lysozyme gene were inoculated by *Erwinia chrysanthemi* and incubated for 2 days.

Table 4 Disease indexes (Black Leg) of transgenic plant lines and non-transgenic cultivars.

Line	Disease index (Average ± SE)
R93	2.00 ± 0.58 a
May-queen	3.33 ± 0.33 b
Danshakuimo	3.50 ± 0.50 b
EG3	4.00 ± 0.00 b

EG3: transgenic plant line using GUS gene (cultivar May-queen). SE stands for standard errors. Data followed by the same letter are not different significantly at 5% level.

and R95 showed significantly low disease indexes and that of R93 was more stable within replicates (Figure 4, 5). Tubers of R93 sowed again and larger tubers of R93 were harvested. They were assayed again and disease index was significantly lower than those of other line and cultivars (Table 4).

Growth of disease resistant lines

Growth of transgenie lines was compared to that of their non-transgenic cultivars in the isolated green house. No differences were observed in shape of leaves, plant heights and other charaeters of their appearance (Data not shown). Tubers harvested from these plants individually and it was confirmed transgenic plants produced similar tubers to those of non-transgenie plant (Table 5, Figure 6, 7).

Table 5 Tuber characters of transgenic plant lines and non-transgenic cultivars.

Name of line or cultivars	Numbers of tubers/plant	Average weight of tuber (g)
R93	13	84.7
Danshakuimo	7	186.6
Name of line or cultivars	Numbers of tubers/plant	Average weight of tuber (g)
EC100	24	33.8
May-queen	21	35.4

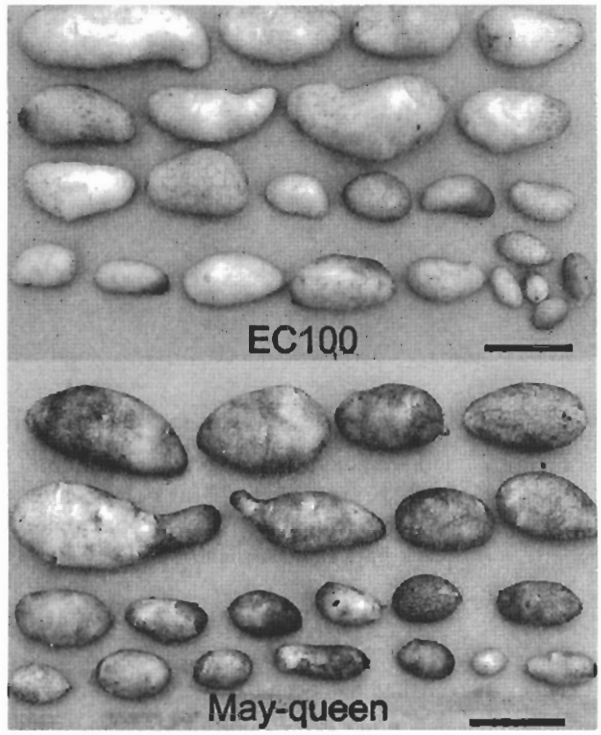

Figure 6 Tubers harvested from transgenic line EC100 resistant to Dry Rot and non-transgenic cultivar May-queen. Black bars indicate 5cm.

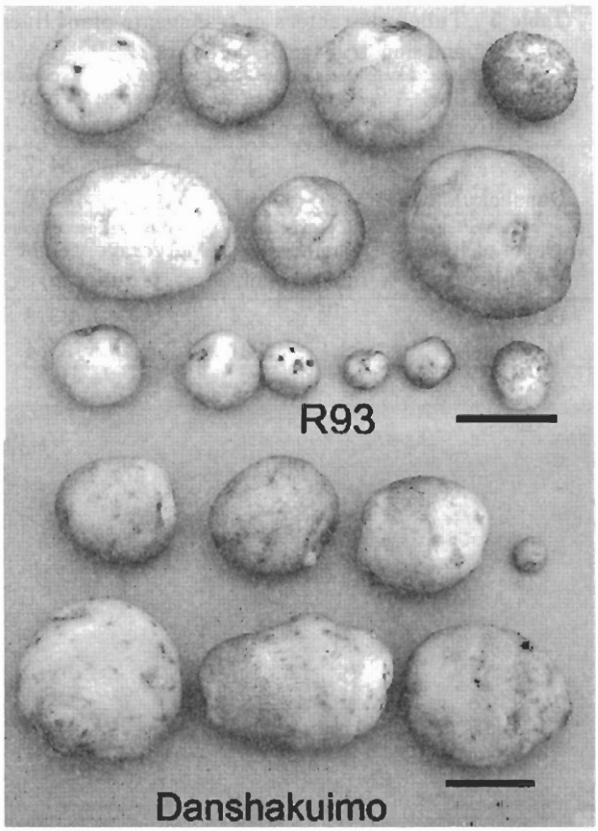

Figure 7 Tubers harvested from transgenic line R93 resistant to Black Leg and non-transgenic cultivar Danshakuimo. Black bars indicate 5cm.

Discussion

Tuber disc method has been used for transformation of potato because tuber discs could produce morphologically normal shoots without intermediary callus formation and because tubers were easy to store and to handle a lot of materials for transformation trials[13]. In this study, the wild spinach chitinase gene and the chicken lysozyme gene were also successfully introduced into potato by tuber disc method and no morphological abnormalities were observed in transgenic plants. Although slight modification such as use of other antibiotics in selection medium was necessary and the rates of shoot regeneration varied in cultivars using this culture conditions, such as concentration of plant growth regulators, this method is one of logical ways for transformation of potato.

The wild spinach chitinase gene was transformed with E12Ω promoter which had two extra-enhancer sequences and 10 times higher expression of the marker gene was observed compared with CaMV35S promoter in the transgenic tobacco[6]. Disease indexes (Dry Rot) of transgenic potato lines harboring the same gene with CaMV35S as promoter were also assayed but the averages of disease indexes of each line were almost same as that of lines containing the same gene and E12Ω as promoter (Data not shown). Although more studies were necessary, E12Ω promoter might not enhance the marker gene expression in potato to the same level as tobacco.

Disease indexes of R93 (the chicken lysozyme gene) and EC100 (the wild spinach chitinase gene) were compared and significantly lower than not only those of *in vitro* non-transgenic cultivars (treated control) and transgenic line harboring GUS gene (Table 3, 4) but also those of non-transgenic cultivars grown in the field (Data not shown). Thus, the origin of these resistances against each disease was not somaclonal variations or mutation during transformation but the effects of the transgenes used in this study.

Effect of the wild spinach chitinase on the resistance against the fungal infection has been explained by fungal cell wall lysis by the chitinase or by producing cell wall degradation substances draw out the ability of defense mechanisms from transgenic plants. In strawberry, Ikeda et al.[3] reported the effects of exogenous microbial chitinase on the digestion of haustoria of *Sphaerotheca humili* and Asao et al.[1] produced resistant strawberry against *S. humili* by the introduction of the rice chitinase gene. This trausgenic strawberry showed little lesion area caused by the inoculation of *S. humili* on their leaves. In this study, hyphae of *Fusarium avenaceum* (Dry Rot) on the tuber discs of EC100 did not grow or grew considerably slower than those on tuber discs of non-transgenic cultivar and of transgenic line harboring GUS gene. Thus, effects of the wild spinach chitinase gene introduced into EC100 might be the same as the rice chitinase gene in strawberry.

The chicken lysozyme gene highly homologous to HEWL[14] was successfully isolated from oviduct of 'White leghorn' and introduced into potato. Introduction of the human lysozyme gene into tobacco generated transgenic plants with disease resistance against both phytopathogenic bacteria and fungi. The chlorotic halo of the disease symptoms by *Pseudmonas syringae* was strongly reduced in the transgenic tobacco plants[8]. The chicken lysozyme gene is highly homologous to the human lysozyme and they can degrade both bacterial and fungal cell walls[2,9]. Iu this study, one of 9 trans-

genic lines, R93 showed significantly resistance against Black Leg (Table 2). The degrees of rottenness of the inoculated tuber discs were strongly suppressed in those of R93. Thus, the chicken lysozyme gene introduced into R93 might work as same way as other lysozyme genes reported before.

R93 and EC100 grown in the isolated green house showed no differences in characters of their appearance and tubers compared to their non-transgenic cultivars. Thus, these lines had disease resistance by transformation of foreign gene and maintained other characters of their non-transgenic cultivars.

Many pest resistant lines of potato developed by genetic transformation technique were reported and several of them have been released as commercial uses since 1995[13]. Unfortunately, genetically modified food has been met with strong oppositions from consumers especially in Japan and Europe. Thus, it is impossible for us not only to perform some experiments in the field but also to release these newly developed potato lines resistant to fungal or bacterial diseases now. Genetic engineering is a promising technique to produce new cultivars which are impossible or time-consuming to make by conventional breeding methods. For further development and utilization of genetically modified plants, scientists are obliged to diffuse their knowledge into the people.

References

1) Asao, H., Nishizawa, Y., Arai, S., Sato, T., Hirai, M., Yoshida, K., Shinmyo, A., Hibi, T. "Enhanced resistance against a fungal pathogen *Sphaerotheca humuli* in transgenic strawberry expressing a rice chitinase gene". Plant Biotech., **14(3)**, 145-149 (1997).

2) Düring, K. "Can lysozyme mediate antimicrobial resistance in plant?". Plant Mol. Biol., **23**, 209-214 (1993).

3) Düring, K., Porsch, P., Fladung, M., Lörz, H. "Transgenic potato plants resistant to the phytopathogenic bacterium *Erwinia carotovora*". Plant J., **3**, 587-598 (1993).

4) Ikeda, S., Toyoda, H., Yoshida, K., Koreeda, K., Chatani, K., Ouchi, S. "Digestion of haustoria of *Sphaerotheca* powdery mildew fungi by an exogenous chitinase". Ann. Phytopathol. Soc. Japan, **58**, 780-783 (1992).

5) Kikuta, Y., Okazawa, Y. "Control of root and shoot-bud formation from potato tuber tissue culture *in vitro*". Physiol. Plant., **61**, 8-12 (1984).

6) Mitsuhara, I., Ugaki, M., Hirochika, H., Ohshima, M., Murakami, T., Gotoh, Y., Katayose, Y., Nakamura, S., Honkura, R., Nishiyama, S., Ueno, K., Mochizuki, A., Tanimoto, H., Tsugawa H., Otsuki, Y., Ohashi, Y. "Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants". Plant Cell Physiol., **55**, 1067-1077 (1996).

7) Murashige, T., Skoog, F. "A revised medium for rapid growth and bioassays with tobacco tissue culture". Physiol. Plant., **15**, 473-497 (1962).

8) Nakajima, H., Muranaka, T., Ishige, F., Akutsu, K. "Fungal and bacterial disease resistance in transgenic plants expressing human lysozyme". Plant Cell Rep., **16**, 674-679 (1997).

9) Sambrook, J., Fritsch, E. F., Maniatis, T. "Molecular cloning: A laboratory manual/ second edition 1-3". Cold Spring Harbor Laboratory Press, New York (1989).

10) Sawada, H., Tsuchiya, K. "Taxonomy of the genus Agrobacterium". Jpn. J. Phytopathol., **69**, 349-365 (2003).

11) Sheerman, S., Bevan, M. W. "A rapid transformation method for *Solanum tuberosum* using binary *Agrobacterium tumefaciens* vectors". Plant Cell Rep., **7**, 13-16 (1988).

12) Trudel, J., Potvin, C., Asselin, A. "Expression of active hen egg white lysozyme in transgenic tobacco". Plant Sci., **87**, 55-67 (1992).

13) USDA. "Graphs: Field releases/deregulation of transgenic plants". (1999).

14) Watanabe, J. A., Watanabe, K. N. "Pest resistance traits controlled by quantitative loci and molecular breeding strategies in tuber-bearing *Solanum*". Plant Biotech., **17(1)**, 1-16 (2000).

アグロバクテリウム法によりニワトリ由来リゾチーム遺伝子とアカザ由来キチナーゼ遺伝子を導入したバレイショの病害抵抗性の評価

平井 泰[1] 鈴木 孝子[1] 柳田 大介[2] 佐々木 純[1]
楠目 俊三[3] 木口 忠彦[1] 竹内 徹[1] 玉掛 秀人[1]

要 約

ニワトリ由来リゾチーム遺伝子とアカザ由来キチナーゼ遺伝子をアグロバクテリウム法を用いてバレイショに導入した。アグロバクテリウムを接種した塊茎ディスクを0.1mg/lのIAA，GA_3及び0.5mg/lのzeatinを含む改変ホワイト培地で2日間共存培養後，200mg/lカーベニシリンと100mg/lカナマイシンを含む同培地に移植した。得られた個体の頂芽節を100mg/lカナマイシンを含む1/2MS培地に移植し，発根した個体のマイクロチューバーを養成すると同時に，導入した遺伝子の存在と発現を確認した。遺伝子導入を確認できた個体から隔離温室内で塊茎を養成し病害抵抗性試験に供試した。アカザ由来キチナーゼ遺伝子を導入した系統からは乾腐病（*Fusarium avenaceum*）に抵抗性の「EC100」を，ニワトリ由来リゾチーム遺伝子を導入した系統からは黒あし病（*Erwinia chrysanthemi*）抵抗性の「R93」を選抜した。これらの系統は原品種と同様の生育を示した。

1 北海道立中央農業試験場，069-1395，夕張郡長沼町
E-mail:hiraidai@agri.pref.hokkaido.jp
2 同上（現：北海道立北見農業試験場，099-1496，常呂郡訓子府町）
3 同上（現：北海道立上川農業試験場，078-0397，比布町）