Agrobacterium Mediated Transformation with Antibacterial Genes for Controlling Bacterial Wilt (*Pseudomonas solanacearum*) on Tetraploid Potato Varieties

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Abstract

Seven tetraploid potato genotypes were employed to put artificial antibacterial genes against bacterial wilt (*Pseudomonas solanacearum*). The antibacterial genes derived from giant silk moth (*Hyalophora cecropia*). These genes are associated with the antibacterial peptides, cecropin, attacin, lysozyme or shiva. These genes were constructed with either cauliflower mosaic virus (CaMV35S) or wound-inducible (WI) promoter, and with franking gene markers in a binary vector pBI121. *Agrobacterium rhizogenes* (R1000) which contains the binary vector was used to transform the potato genotypes. Hairy roots obtained were cultured in MS medium with Claforan then, in a regeneration medium containing 100ppm kanamycin. Regenerated plantlets were evaluated for the expression of flanking marker genes: kanamycin resistance and GUS reaction. After selection, those plantlets were used for Southern hybridization to confirm the presence of the target gene(s) for antibacteria. Phenotypic resistance evaluation on them was made using *Pseudomonas solanacearum*, Race 3, Biovar II, CIP isolate 204 at greenhouse. Some improvement on the level of resistance was seen on regenerates from three original genotypes, while there was variation on the level of resistance among regenerates from the same original genotype. RT-PCR revealed substantial level of gene expression, however, the amount of resulting lytic peptides was not detected well by ELISA. Thus, it was speculated that a post-transcriptional degradation and /or a post-translational degeneration by endogenious xenobiotic metabolizing mechanisms or endogenous proteinases.

Key words: Potato, bacterial disease, antibacterial lytic peptides, resistance, *Agrobacterium rhyzogenes*

Introduction

Bacterial wilt caused by *Pseudomonas solanacearum* is a devastating disease especially in tropical and subtropical regions over the world, and severe infection in potato field could result in 0 % crop yield (French 1985). Two major groups of *P. solanacearum*, Biovar I (race 1) and Biovar II (race 3) cause the disease in potato crops, while race 1 regularly identified in low land tropics, and race 3 is seen rather in subtropical and high land tropics (French 1985).

Breeding effort has been made to generate potato varieties with resistances to bacterial wilt (Schmiediche 1988, Watanabe *et al.* 1992). Wild relatives of cultivated potatoes have high level of resistance to the disease, which did not exist in cultivated potatoes (Watanabe *et al.* 1995). The resistances derived from diploid wild species were used to generate tetraploid breeding lines to be used as parents in breeding, and significant improvement in resistances to bacterial wilt was seen in tetraploid breeding population (Watanabe *et al.* 1995). However, the resistances available in the present tetraploid cultivars are not yet sufficient even with integrated management practice against the disease (Watanabe *et al.* 1999).

Lytic peptide genes derived from giant silk moth (*Hyalophora cecropia*) are effective to

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various bacterial and fungal species (Boman *et al.* 1985, Jaynes *et al.* 1987, Jaynes 1989, Trinca *et al.* 1991). These lytic petide genes are specific to prokaryotes with limited effective range of bacterial and fungal species, and no toxic effect to eukaryotic cells (Jaynes *et al.* 1987). Biochemically different groups of lytic petides were derived; these are cecropin, attacin, and lysozyme. The genes confer the function to produce the lytic peptides were isolated and constructed into Ti plasmids which would enable the transfer of the genes into potato chromosomes via Agrobacterium infection (Jaynes *et al.* 1987, Trinca *et al.* 1991).

Agrobacterium rhizogenes mediated transformation of plant species is one of powerful tools to transfer foreign genes of interest into target plant chromosomes (Dobigny *et al.* 1996, Hooykaas 1989, Ooms *et al.* 1985, 1987, Tepfer and Casse-Delbart 1987). Transformation system by hairy root formation infected by *A. rhizogenes*, has been established well on potato (Visser et al. 1989a, Wenzler et al. 1989), and achievements have been demonstrated to improve potato cultivars such by insertion of coat protein genes from potato virus X and potato leaf roll virus (Jongedijsk *et al.* 1992, van der Wilk *et al.* 1991).

In this paper, we report transformation on tetraploid potato varieties with antibacterial gene constructs and the level of resistance in transgenic potato plants against bacterial wilt, Biovar II race 3.

Materials and Methods

1. Potato genotypes

Seven tetraploid potato genotypes are used for the experiment (Table 1). These are cultivars or are used as parental lines in breeding worldwide through International Potato Center, especially in tropical and subtropical areas. All of these are susceptible to bacterial wilt, while have some other important traits such as virus resistances and adaptation to tropics.

Table 1. List of tetraploid potato breeding lines used in the transformation with *Agrobacterium rhizogenes*, R1000.

Genotype	Female parent	Male parent	Susceptability to Bacterial wilt
Achirana.INTA	MPI61375.23	B25.65	S
LT-8	LT-1	PVY+PVXbulk	S
LT-9	LT-1	PVY+PVXbulk	S
Desiree	Urgenta	Depesche	S
86007	Achirana.INTA	7XY.1	S
86017	Achirana.INTA	7XY.1	S
86056 (B71.240.2)	Achirana.INTA	7XY.1	MR

2. Gene constructs, vector and *Agrobacterium*

Origin and description of the genes constructs, the plamid, and *Agrobacterium rhizogenes* are stated in CIP(1991), Jaynes *et al.* (1987), Jefferson (1987) and Visser *et al.* (1989a). The genes which confer lytic peptides, attacin (*Att*), cecropin 38 (*C38*), and lysozyme (*Lys*) were isolated from giant silk moth (*Hyalophora cecropia*) (Boman *et al.* 1985, Jaynes *et al.* 1987, Jaynes 1989). These genes were attached to cauliflower mosaic virus 35S (CaMV35S) double promoter. Cecropin 38 (*C38*) and Attacin (*Att*) genes were also attached to Wound Inducible (*WI*) one. Chicken lysozyme gene (*Chly*) attched with CaMV35S promoter was also used for the comparison. The antibacterial genes are franked by two marker genes;

kanamycin resistance and γ-glucuronidase (GUS) genes (CIP 1991, Jefferson 1987). These constructs are vectored by a binary vector plasmid pBI121 in *Agrobacterium rhizogenes*, R1000. The binary vector pBI121 was used as control (empty plasmid) without putting target genes to see the efficiency of transformation.

3. Transformation procedures

General procedures on inoculum preparion and inoculation of *Agrobacterium* is referred to Visser *et al.* (1989a). Fourty plantlets of each potato genotype with construct combination were inoculated. Four platlets were grown in each magenta box with total of ten boxes per construct x potato genotype combination. Internodes of living in vitro plantlets were injured by scalpel and bacterial suspension was put to the wounded part of the internodes. Symptom of infection of the bacterium usually showed up in two weeks with development of hairy roots at the infected region.

Hairy roots were harvested and cultured in MS medium with 200 ppm of Claforan to kill Agrobacterium, then the hairy roots were placed on regeneration medium CD (Espinoza and Dodds 1985) with 100 ppm of kanamycin. Plantlets regenerated from CD medium were placed in MSA medium (Espinoza and Dodds 1985) for rooting, while testing for kanamycin resistance as screening for one of franking markers. Then, kanamycin resistant plantlets were tested for another marker GUS (β-glucuronidase). Roots from the kanamycin resistant plantlets were placed ELISA mini titer plate with 50 micro liter each of X-glucuronidase. Roots with blue color reaction were regarded as GUS positve, which indicate presence of GUS marker gene.

4. Identification of transgenic plants with southern hybridization

General procedures on DNA isolation, digestion with restriction enzyme, agarose electrophoresis, southern blotting, and autoradiography are followed after Sambrook et al. (1989). DNA were isolated from the candidate transgenic plants which were selected for GUS and kanamycin resistance genes, and digested with HindIII. Ten micro gram of the digested DNA was loaded to each lane of agarose gel for electrophoresis in order to estimate the copy number of inserted genes, which would be determined by the density of signals from autoradiogram. Genescreen nylon filter was used for southern hybridization.

5. Inoculation of *Pseudomonas solanacearum* which causes bacterial wilt

Inoculation procedures are referred to El-Nashaar *et al.* (1990) using Pseudomonas solanacearum, Biovar II, race 3, CIP isolate 204. The CIP isolate 204 is the known most virulent isolate and used effectively for screening for the resistance to bacterial wilt caused by race 3 (El-Nashaar *et al.* 1990, Watanabe *et al.* 1992). Forty plants from each regenerate were propagated and inoculated with the inoculum and evaluation was made for scoring 0-4 (no wilt to complete wilt) (El-Nashaar *et al.* 1990).

Results and Discussion

1. Transformation: efficiency and occurrence of transgenic plants

It seems that efficiency in regenerating hairy root was not high, and even among genotypes with regeneration showed variation in time taken for regeneration (Table 1). Only potato genotype 86007 showed regeneration of hairy root after inoculation of the bacterium to all constructs with CaMv35S promotor, while, its full sibs, 86017 and 86056 did not show hairy root formation except 86017 with *Chly* gene. Furthermore, one of their parent Achirana.INTA did not show any regenration of hairy root. The low efficiency of regeneration of the hairy root may be related to heritable tissue culture ability in potato genotypes (Orrillo and Watanabe 1995, Singsit and Veilleux 1989), which may be caused by the parent, Achirana.INTA. Although only four combination of potato genotype with *WI* promotored constructs were tested, LT-9 and Desiree showed responce to the infection of the bacterium with *C38* gene with *WI* promotor.

Enhancment of the infection with *Agrobacterium rhizogenes* could be achieved by

treatment of phenol like substances or acetosiringon (Tepfer and Casse-Delbart 1987) which are recognized by the receptor of the bacterium to initiate and accelerate the function of the vir genes of Ti plasmid. As it seemed that some potato genotypes had inability in tissue culture competence in this experiment, some technical improvement is essential to transform various potato genotypes.

When infection of the bacterium occurred and hairy roots were formed, all potato genotypes with such hairy roots resulted in at least some GUS positive plantlets (Tables 1 and 2), which could indicate the successful transformation with the target gene construct. Only potato genotype 86007 resulted in many plantlets with GUS positive reaction, while LT-9 and Desiree had a few plantlets with GUS positive reaction. With the southern hybiridization using an antibacterial gene as probe, the majority of GUS postive plantlets were confirmed as conferring the corresponding gene.

Table 2. Numbers of regenerated plantlets, GUS positive individuals and the positive clones with Southern hybridization in the seven 4x potato genotypes. (# Regeneration / # of GUS positive / # Southern positive)

Genotype	Achirana	LT-8	LT-9	Desiree	86007	86017	86056
Constructs[a]							
Ca2 Att	No	No	No	No	42/29/27	No	No
Ca2 C38	No	No	No	No	58/49/48	No	No
Ca2 Chly	No	No	No	No	36/34/33	No	No
Ca2 Lys	No	No	No	No	40/37/35	2/0/0	No
Ca2 empty	No	No	No	No	7/ 5/5	No	No
WI Att	--	--	No	No	--	--	--
WI C38	--	--	5/2/2	3/1/1	--	--	--

[a] Gene abbreviations: *Ca2*: cauliflower mosaic virus C35S promotor, *Att*: attacin. *C38*: cecropin 38, *Chly*: chicken lysozyme, *Lys*: lysozyme, *WI*: would inducible promotor, respectively.

[b] No: No regenerates, --: untested, respectively.

In overall, the obtention of the transgenic plants were not very high (Table 1), however, southern hybridization indicated strong signals of the presence of the target constructs in these transgenic plantlets (Table 2 and Photo not shown). So far as transgenic plants always contain the target gene(s) and desirable agronomic traits, the efficiency in transformation may not be so important, but it would be often anticipated that somaclonal variation via transformation likely occur (Brown et al. 1991, Dale and Hamson 1995, Dale and McPartlan 1992, de Vries-Uijtewaal et al. 1989; van den Bulk 1991), thus, fair amount of transgenic plantlets per original potato genotype would be required for selecting desirable regenerate(s) with the resistance gene and agronomic traits.

2. Phenotypic resistance in regenerates

Figures 1 and 2 shows the result of inoculation of *Pseudomonas solanacearum* which is the causal agent of the bacterial wilt. There was variation in susceptability to the pathogen among different regenerates from the same original potato genotype (Fig. 1). As overall, a slight improvement in resistance was seen (Fig. 2), on the other hand, extremely strong resistance was not observed although it was anticipated from the previous in vitro testing (Jaynes *et al.* 1987). Whereas no fair comparison was not made on all possible construct x potato genotype combinations, it appears that *WI* promotor may have better contribution

toward the expression of the target antibacterial genes (Fig. 2). RT-PCR revealed substantial level of gene expression (Data not shown), however, the amount of resulting lytic peptides was not detected well by ELISA using the peptide associated polyclonal antibody (data not shown). Thus, a post-transcriptional degradation and/or a post-translational degeneration by endogenious proteinases (Baulcombe 1996).

There are several issues to be discussed and elucidated with respect to expression of the target genes in potato plants. These are in the followings: a) effect of inserts vs genes, b) copy number of the target genes, c) position effect of the inserted genes, d) stability of the inserted genes in mitosis and vegetative propagation, e) translational and transcriptional modification, f) methylation of the genes, and g) effect of promotors (Baulcombe 1996, Dobigny et al. 1996, Inui et al. 1998, Suzuki *et al.* 1997).

The methylation of the inserted genes would influence the expression, as Ottaviani *et al.* (1993) reported the presence of the methylation in transgenic potatoes and it altered the exression of the genes. Furthermore, low expression of genes were seen at the methylated genes (Doefler 1983, Ngernprasirtsiri *et al.* 1988).

Selecting proper promotors could alter the expression of the inserted genes as seen in WI promotor (Fig. 2). Different type of promotors could have different levels of expression of the inserted genes (Comai et al. 1990, Ishige et al. 1991, Vancanneyt *et al.*. 1990, Visser *et al.* 1989a). Thus, Reconstruction of genes or change in promotor regions could have more opportunity to obtain transgenic plants with high gene expression.

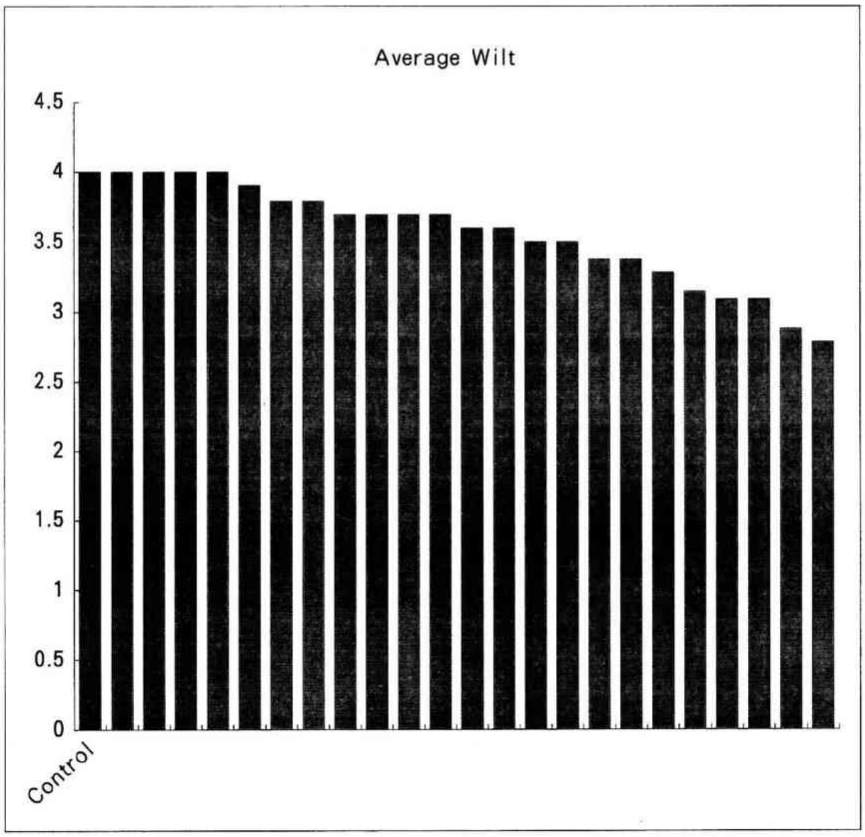

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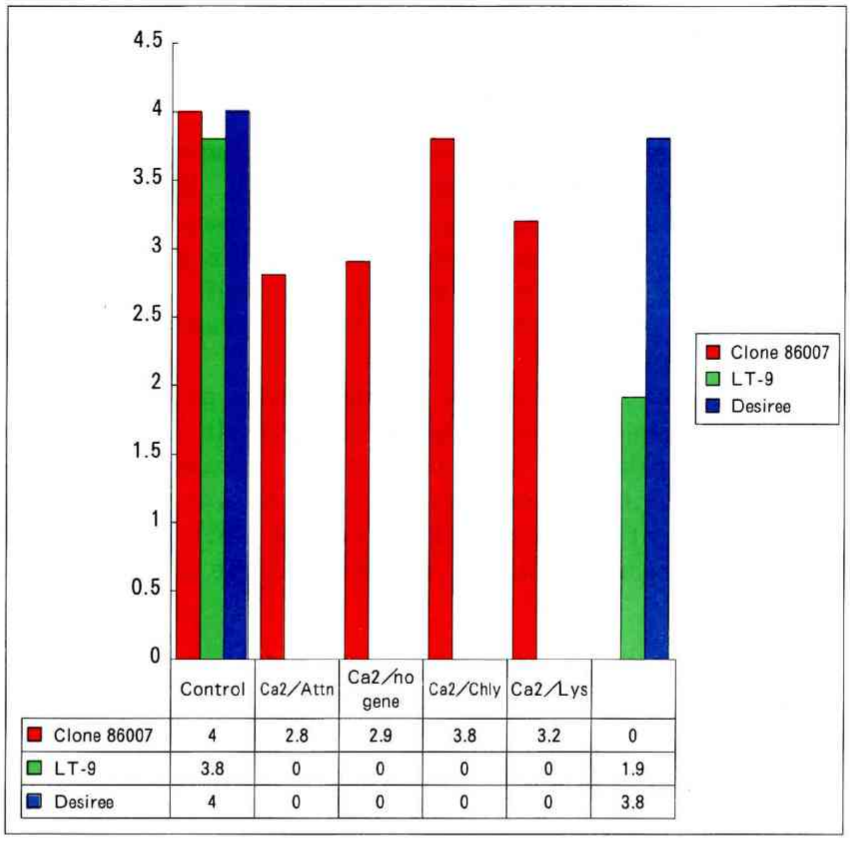
Fig. 1. Variation in bacterial wilt susceptability in transgenic plants from 86007 using a gene construct *C38* with *CaMV35S* promotor. Each bar indicates average susceptability of 40 plants from each individual transgenic regenerate. 0 - 4 scores indicate 0 (no wilt) to 4 (complete wilt).



	Control	Ca2/Attn	Ca2/no gene	Ca2/Chly	Ca2/Lys	
Clone 86007	4	2.8	2.9	3.8	3.2	0
LT-9	3.8	0	0	0	0	1.9
Desiree	4	0	0	0	0	3.8

Fig. 2. Bacterial wilt susceptability in trasngenic plants from three potato genotypes with different gene constructs. Each bar represents the level of the susceptibility in the most resistant regenerate (Average of 40 plants as shown in Fig. 1) derived from the same construct. Different bar indicates the level of the susceptibility on the different construct. Refer to Fig. 1 for scaling 0-4.

Furthermore, questions would be placed on: a) transmission of the inserted genes by sexual crosses (Brown *et al.* 1991, Visser *et al.* 1989b), and b) its stability in the progeny (de Vries-Uijtewaal *et al.* 1989), c) field performance of the resistance and the agronomic traits (Dale and McPartlan, 1992; Jondgedijk *et al.* 1992), and d) duration of the resistance. These issues are important to use the transgenic plants as potato cultivars since the stability of the resistance is essential for cultivar development.

The following issues should also be considered as the system used tissue culture derived transgenic plantlet after hairy root selection, 1) Change in chromosome number, 2) structural change in chromosomes, 3) mutation in loci, 4) mitotic crossing over, 5) change in expression of host plant (potato) genes, 6) change in copy number of genes, 7) transposon activation, and 8) mutation in organelle DNAs. These concerns should provide more information in understanding the nature of variation in the phenotypic expression in the transgenic plants.

It seems that the antibacterial genes could improve tetraploid potato genotypes via transformation as shown in Figs. 1 and 2. This would provide a potential to improve potato cultivars adapted to tropical which need to have bacterial wilt resistances, on the other hand, technical improvement is essential for efficient transformation and gene expression. A further effort to re-design the lytic peptides could increase efficiency in anti-

bacterial effect and specificity to the pathogen ranges (Jaynes *et al.* 1993), and what is more could provide the compatibility with the endogenous metabolic arrays, which does not degrade the lytic peptide products for endogenous protection mechanism (s).

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This paper was dedicated to Ms. Carmen Següeñas who passed over due to a sudden brain disease. The original research was achieved in the early 1990 during the turmoil due to political and social difficulties at the host country. Because of the disservice by the terrorism to the members of this research group, the data was temporally lost and it was unable to publish this paper at the time. With the help of BOST Institute at Kinki University to finalize the missed data using its facility, we could achieve the initial goal by materiali-z ing the publication.

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摘　要

青枯病（*Pseudomonas solanacearum*）抵抗性付与のための抗細菌性遺伝子の *Agrobacterium* 法による4倍体バレイショ品種への形質転換

渡邊和男、V. Zambrano、J. Benavides、C. Sigüeñas、H. El-Nashaar、J. H. Dodds

バレイショ品種における青枯病（*Pseudomonas solanacearum*）に対する抵抗性を飛躍的に向上させるため、カイコ由来の溶細菌性タンパクを生産する遺伝子群を用いた。溶細菌性遺伝子を含む binary vector をそれぞれ導入した *Agrobacterium rhizogenes*, R1000を用いて、バレイショ品種に遺伝子の導入・発現を試みた。組み換えバレイショ系統は多数得られたものの、閉鎖系温室での抵抗性の程度は組み換え個体間で大きな違いがあり、遺伝子構築や発現様式の調整が今後必要であることが解った。