식물조직배양학회지 제24권 제5호
Korean J. Plant Tissue Culture
Vol. 24, No. 5, 313~317 (1997)

Development of Potato Virus Y-Resistant Transgenic Potato

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감자 바이러스 Y 저항성 형질전환 감자 개발

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Leaf segments of the potato (*Solanum tuberosum* L.) genotypes, ND860-2, Norchip, Russet Norkotah, Goldrush, and Norqueen Russet were transformed with the coat protein gene of potato virus Y (PVY). The white-skinned genotypes, ND860-2 and Norchip, were easily transformed and regenerated into shoots, whereas the three russet-skinned genotypes had low frequencies of regeneration. Transformed shoots were generally recovered in four to six weeks. Antibody to PVY coat protein detected a single band of 30 kD in western blots of transgenic plants. Transformed plants had a normal phenotype in the greenhouse and many showed a delayed buildup of PVY following inoculation. Several transgenic lines had negative ELISA readings 85 days after inoculation. Transgenic lines which did not show detectable levels of PVY antigen will be further tested for resistance to PVY.

key words : *Agrobacterium*-mediated transformation, virus resistance

INTRODUCTION

Viral diseases constitute a serious threat to cultivation of potato, a vegetatively-propagated crop. Potato virus Y (PVY) became an important concern to potato seed growers. Because of PVY infection, the percentage of seed potato lots that were rejected for seed certification has been increased. This virus epidemic resulted in substantial economic loss and restricted seed availability for certain cultivars in subsequent growing seasons. Growers would benefit from virus resistance but would prefer to see this trait in already proven cultivars, rather than wait for resistant breeding lines which must still be evaluated for many other important traits (Hoekema et al., 1989: Lawson et al., 1990: Powell et al., 1986). The process of traditional breeding methods is complicated by tetrasomic segregation patterns and incomplete fertility in many tetraploid commercial cultivars including potatos. Recently, genetic engineering techniques, such as *Agrobacterium*-mediated DNA transfer, have great potential to improve established cultivars by introducing genes of interest without altering commercially desirable phenotypes (Hoekema et al., 1989: Ooms et al., 1987: Sheerman and Bevan, 1988). This study was undertaken to develop transformation systems for 5 different potato cultivars and to transform these cultivars with the CP gene of PVY to confer virus resistance..

MATERIALS AND METHODS

Plant Material

The potato genotypes, ND860-2, Norchip, Russet Norkotah, Goldrush, and Norqueen Russet were obtained from the North Dakota State Seed Department as pathogene-tested in vitro plantlets. These source plants were propagated aseptically by single node cuttings in 25 mm×100 mm culture tubes in medium containing MS basal salts

(Murashige and Skoog, 1962), 20 g/L sucrose, 0.2 mg/L GA_3, 0.05 mg/L NAA, 2.0 mg/L calcium pantothenate, and 8 g/L agar (Difco). Cultures were subcultured by transferring nodal segments to fresh medium every 2 months.

Bacterial Strains

The PVY^O CP gene was obtained from P. Berger, University of Idaho, Moscow, ID, cloned as a 5'-*Hind* Ⅲ and 3'-*Sac* I fragment in binary vector plasmid pKYLX7 (Schardl et al., 1987). This binary vector contained the neomycin phosphotransferase (NPT Ⅱ) gene which confers kanamycin resistance. Transfer into *Agrobacterium tumefaciens* strain LBA 4404 was performed via triparental mating utilizing helper plasmid pRK 2013. Selected colonies were grown in 5 ml of LB liquid medium containing 50 mg/L kanamycin and 200 mg/L streptomycin on a shaker at 28°C for 48 h, then centrifuged and resuspended in fresh medium for the plant inoculation.

Transformation, Selection, and Regeneration

Small leaf segments (~5 mm) were cut from in vitro plants and wounded with a sterile razor blade. These wounded leaf segments (50 segment per genotype) were swirled in the *Agrobacterium* solution, blotted on sterile filter paper, and inoculated on a non-selective regeneration medium containing MS salts, 30 g/L sucrose, 4 mg/L zeatin riboside and 3.5 mg/L IAA (Park et al., 1995). After 3 days co-cultivation on the dark at 22°C, inoculated leaf segments were transferred to a selective regeneration medium, which contained, in addition to the non-selective medium, 200 mg/L cefotaxime and 25 or 50 mg/L kanamycin. After 10 additional days of dark treatment, the culture plates were transferred to an incubation room and distributed randomly. The photoperiod was 16 h, provided by cool white fluorescent lamps (54 μmol photons $m^{-2}s^{-1}$): incubation temperature was approximately 24°C. After two weeks in the light, the leaf segments were transferred to the second selection medium, which consisted of the regeneration medium plus 500 mg/L carbenicillin and 50 mg/L kanamycin. The number of segments forming callus, the number of segments producing one or more shoots, and the number of shoots exceeding 5 mm were recorded.

Confirmation of Transformation

Regenerated shoots from the selective medium were excised and placed on rooting medium containing MS basal salts, 15 g/L sucrose, 0.1 mg/L IAA, 0.01 mg/L kinetin, 7 g/L agar, 500 mg/L carbenicillin, and 100 mg/L kanamycin. Shoots that survived and developed roots after 3 weeks were recorded as putative transformants. Transformants were verified by dot blot hybridization and Southern analysis (Southern, 1975). DNA was isolated from leaf tissue (Shur et al., 1983), transferred to a nylon membrane and probed with α-^{32}P labeled 5'-*Hind* Ⅲ and 3'-*Sac*I fragment in pKYLX7 plasmid DNA. Random primer labeling was conducted using the method of the GIBCO BRL kit (Life Technologies, Inc., Gaithersburg, MD). Prehybridization, hybridization, washing, and autoradiography were performed according to Geoffrey et al. (1981).

Plant Virus Resistant Test

Six in vitro plantlets of each mother genotype (control) and in vitro plantlets from each independent transformant recovered from that genotype were planted into 6-pak trays with a commercial peat-lite mix (Sunshine Mix #1, Fisons Horticulture Inc, Bellevue, WA 98004) and placed on a bottom-heated bench in a greenhouse mist chamber for 1 week. Established plants were transplanted into 10 cm pots in the same soil mix, randomized, and placed on a greenhouse bench. Natural light was supplemented by metal halide lamps that provided a 16 h photoperiod with a flux density of photosynthetically active radiation of 150 μmol photons $m^{-2}s^{-1}$ at the top of the plants. The plants were fertilized with N-P-K(15:16:17) at every watering and grown to maturity. Plant height was recorded prior to senescence: harvested tuber number and weight were recorded after senescence. In a second experiment, a similar set of transformed and control plants of the same genotypes were infected with PVY by dusting leaves of young plants (4 to 6 leaf stage) with carborundum and rubbing the leaves two times with a 1:20 diluted solution of inoculum from young PVY infected tobacco leaf tissue extracted in 50 mM potassium phosphate with 10 mM NaEDTA, pH 7.0. Virus Levels were determined by ELISA (enzyme-linked immunosorbent assay) at 15, 23, and 30 days after infection, using the method described by ELISA kit (Agdia, Inc., Elkhart, IN). Positives were identified as a yellow color and negative as colorless. Plants with negative readings after 30 days were again at 85 days after infection. Differences between sets of controls and transformed types were determined by analysis of variance.

Immunoassays

Double-sandwich ELISA for PVY was performed using a commercial assay (Agdia, Inc., Elkhart, IN) and quantified at 405 nm. Readings greater than 0.100 absorbance units were considered positive. Western blots were prepared according to Burnette (1981). Fresh leaf samples (100 mg) were extracted in 1 ml of reducing buffer, which contained 65 mM Tris-HCl (pH 6.8), 10% glycerol, 0.1% mercaptoethanol, and 20%(w/v) SDS. Samples were pre-incubated for conducted at 0°C onto a nitrocellulose membrane. Primary antibody (rabbit anti-PVY) and secondary antibody (alkaline phosphotase-conjugated goat anti-rabbit) were purchased from Agdia Inc, Elkhart, IN, and were used at a dilution of 1:1000. Color developing reagents for the alkaline phosphatase reaction were purchased from Bio-Rad Laboratories, Richmond, CA.

RESULTS AND DISCUSSION

The optimum levels of phytohormones and length of dark treatment for regeneration of plantlets were determined in prior experiments (Park et al., 1995). Callus formation of transformed segments on selective medium was detected after two weeks and shoot formation after 4 to 6 weeks on selective regeneration medium. Control leaf segments died within 4 weeks on selective medium. The white skinned genotypes, ND860-2 and Norchip, had more transformed shoots than the russet skinned genotypes, Russet Norkotah, Goldrush, and Norqueen Russet (Table 1). The same trend was observed in other experiments to optimize regeneration systems for these genotypes (Park et al., 1995). Therefore, the low transformation frequencies of the russeted genotypes was related to the difficulty in regenerating those genotypes from callus. The reason for this recalcitrance is not yet understood. In any case, the methods used allowed the recovery of transgenic plants of all genotypes. Dot blot hybridization confirmed the transformation of all but two putative transformants which rooted on the 100 mg/L kanamycin medium. This result indicated that kanamycin at 100 mg/L was sufficient to select transformants. Approximately twice as many shoots were produced by leaf segments cultured on the 25 mg/L kanamycin medium compared to the 50 mg/L kanamycin medium (Table 1). However, survival of the shoots from the rooting medium with 50 mg/L kanamycin was only half that of shoots from the 25 mg/L kanamycin medium. This indicated the likelihood of escapes on regeneration medium with 25 mg/L kanamycin.

Extracts from transgenic PVY-CP leaves were negative in double-sandwich ELISA tests for PVY-CP, indicating either that the amount of PVY-CP expressed in transgenic plants was insufficient to be detected by this method or that this method of ELISA is not suitable to detect PVY-CP. However, the same extracts revealed a single band corresponding to a polypeptide of 30 kD when western blots were probed with the PVY antibody. The relative molecular mass of this protein is consistent with that of the PVY-CP reported by other researchers (Farinelli et al., 1992, Fig. 1).

No aberrant phenotypes were observed in the greenhouse, except for one transgenic plant that had a light yellow leaf color. There was generally no difference between transformants of any genotype and the respective control with regard to plant height, tuber number, or tuber yield (Table 2), although each set of transformants had a non-significant increase in tuber number and yield per plant over its control. Therefore,

Table 1. Transformation of 5 North Dakota potato genotypes with potato virus Y (PVY) coat protein gene.

	ND860-2		Norchip		Russet Norkotah		Goldrush		Norqueen Russet	
	Kanamycin concentration (mg/L)									
Characteristics	25	50	25	50	25	50	25	50	25	50
Total no. of segments inoculated	25	25	25	25	25	25	25	25	25	25
No. of segments forming callus	24	24	24	24	20	17	19	13	9	3
No. of segments forming shoots	18	17	17	17	12	4	8	5	2	1
Shoots per leaf segment[a]	3.4	2.6	1.1	1.0	1.3	1.6	1.4	2.0	1.5	1.0
Shoots rooting on kanamycin	9	23	12	16	3	5	7	10	1	1
Transformed shoots confirmed by dot blot hybridization	9	22	12	15	3	5	7	10	1	1
Transformation frequencies (%)[b]	62	54	16	34	4					

[a]: Total no. of shoots/No. of segments producing 1 or more shoots.
[b]: [No. of transformed shoots confirmed by dot blotting/Total no. of segments inoculated (50)] x 100

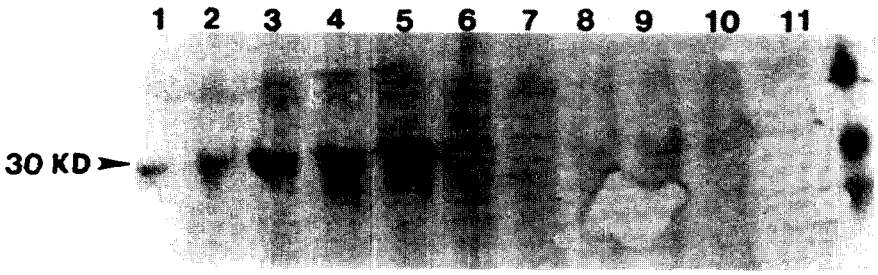

Figure 1. Western blot analysis of PVY CP expression in transformed plants. Protein extracted from leaves was separated on a 10% SDS/polyacrylamide gel and electroblotted on a nylon membrane, and the coat protein was identified by immunoblotting, using anti-PVY. Lanes 1-2: two transformed ND860-2, 3-4: two transformed Norchip, 5-6: two transformed Russet Norkotah, 7-8: two transformed Goldrush, 9-10: two transformed Norqueen Russet, 11: nontransformed ND860-2 (wild type).

Table 2. Plant height, tuber number, and yield per plant of PVY-CP transformed and control plants.

Genotype	Plant height (cm)	Tuber number	Tuber Weight (g / plant)
ND860-2			
PVY-CP transgenic (28)[a]	33.5*	7.3	45.9
Control (6)	40.3	6.2	36.6
Norchip			
PVY-CP transgrenic (27)	27.6	7.9	53.3*
Control (7)	27.6	6.0	35.9
Russet Norkotah			
PVY-CP transgenic (7)	30.0	4.0	13.2
Control (6)	30.4	3.7	14.6
Goldrush			
PVY-CP transgenic (13)	33.9	4.6	23.0
Control (5)	33.0	4.0	20.4
Norqueen Russet			
PVY-CP transgenic (2)	29.0	4.0	21.5
Control (5)	23.7	2.6	14.5
Average			
PVY-CP transgenic (77)	31.0	6.6*	41.0
Control (30)	31.3	4.6	25.1

[a]Number in parenthesis ( ) indicates number of plants tested
*, **indicate significantly different than control at p - 0.1, 0.05, respectively.

Table 3. Percentages of plants with positive PVY ELISA readings at 15, 23, and 30 days after inoculation.

Genotype	Days after inoculation		
	15	23	30
ND860-2			
PVY-CP transgenic (26)[a]	38[b]**	69	92
Control (5)	80	80	100
Norchip			
PVY-CP transgrenic (27)	48**	59**	85
Control (5)	80	80	100
Russet Norkotah			
PVY-CP transgenic (7)	57	71	86
Control (5)	80	80	100
Goldrush			
PVY-CP transgenic (16)	58*	76	100
Control (5)	80	80	100
Norqueen Russet			
PVY-CP transgenic (2)	50	50	100
Control (5)	80	80	100

[a]Number in parenthesis ( ) indicates number of plants tested
[b](No. of plants infected PVY/Total no. of inoculated) × 100
*, ** indicate significantly different than control at p - 0.1, 0.05, respectively.

we did not observe a detrimental effect of transformation on plant performance, in contrast to other studies (Belnap et al., 1994). The present experiment was conducted in the greenhouse and the performance of these transgenic plants has not been tested in the field.

The accumulation of virus in inoculated plants was monitored by ELISA. At fifteen days after inoculation with PVY, about half of the transformants gave positive ELISA readings, compared with 80% of the control plants (Table 3). At 30 day all control plants showed positive for PVY, but seven transformants had negative readings. Therefore, transformation with the PVY-CP gene delayed development of the PVY virus in many transgenic lines. Four of the seven transformed lines which were revealed as negative at 30 day were also negative after 85 day (data not shown). Further testing of these resistant lines, which included one transgenic clone of Russet Norkotah, is underway. Russet Norkotah clones with extreme resistance to PVY would be of particular interest, since this cultivar has been implicated as a symptomless carrier of PVY (Johansen et al., 1988). A few transformed clones had extremely high ELISA readings 15 days after inoculation, indicating more rapid buildup of virus titer than in control plants (data not shown). The mechanism of a quick accumulation of virus in those lines is not known.

The transformation and regeneration system used was efficient, rapid, and allowed recovery of transformed lines of all genotypes, including established cultivars (Norchip, Russet Norkotah, Goldrush, Norqueen Russet), and a breeding line (ND860-2). The rapid recovery of transgenic clones of all genotypes demonstrated the effectiveness of this technology for a breeding program. The white-skinned genotypes had higher frequencies of transformation and regeneration than the

russet-skinned genotype. Many transformants showed a delay in symptom development against infection by PVY and several lines displayed extreme resistance to inoculation in the greenhouse. The low percentage of clones with adequate resistance indicates that a relatively high number of transgenic clones should be screened to recover suitable clones. Lawson et al. (1990) also transformed a major commercial cultivar, Russet Burbank, with the CP genes of PVY. Transgenic plants that expressed CP gene resisted infection by PVY inoculated mechanically. One line was also resistant when PVY was inoculated with viruliferous green peach aphids. These experiments demonstrated that CP protection is effective against mechanical and aphid transmission of PVY.

We recovered one highly resistant line of Russet Norkotah, an important cultivar that has been cited as a symptomless carrier of PVY.

적 요

ND860-2, Norchip, Russet Norkotah, Goldrush 및 Norgueen Russet 등 5개 품종의 감자 잎 조직을 감자 바이러스 Y (PVY)의 외피단백질 (CP) 유전자를 운반하는 *Agrobacterium tumefaciens*을 이용하여 형질전환시켰다. ND860-2와 Norchip 품종과 같은 흰색 감자에서 재분화율과 형질전환율이 높게 나타났다. 형질전환체를 선발한 후 CP의 생성을 western blot으로 확인한 결과 30 kD의 밴드가 나타났다. 온실에서 재배된 형질전환체는 한 개체를 제외하고는 표현형에 있어서는 기존의 품종과 다르지 않았다. 인위적으로 감자 바이러스 Y를 감염시킨 15일 후의 검사결과 control구는 80%의 감염율을 보인 반면 형질전환체들은 38 - 58%의 감염율을 보였다. 30일 후의 검사 결과는 모든 5품종의 control구에서 100%의 감염을 보였으나 형질전환체는 7개체가 저항성을 보였다. 마지막 85일 후의 검사 결과 4개의 저항개체를 발견하였으며 이 개체들은 현재 계속 연구가 진행 중에 있다.

ACKNOWLEDGMENT - We thank Drs. Phil Berger and Jeff Griffin for providing the PVY CP gene in plasmid pKYLX 7. This work was supported by USDA-CSRS grant #92-34297-6959.

REFERENCES

Belnap WR, Corsini D. Pavek JJ, Snyder GW, Rockhold DR, Vayda ME (1994) Field performance of transgenic Russet Burbank and Lemhi Resset potatoes. Am Potato J **71**: 285-296

Burnette WN (1981) Western blotting; electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Bioch **112**: 195-203

Farinelli L, Malnoe P, Collet GF (1992) Heterologous encapsidation of potato virus Y strain O (PVY^O) with the transgenic coat protein of PVY strain N (PVY^N) in *Solanum tuberosum* cv. Bintje. Bio/Technology **10**: 1020-1025

Geoffrey MW, Estelita O, Meinkoth J, Rodrigo F, Barinaga M (1981) Methods for the transfer of DNA, RNA and protein to nitrocellulose and diazotized paper solid supports. Schleicher & Schuell, Inc., Keene, NH

Hoekema A, Huisman MJ, Molecdijk L, van den Elzen PJM, Cornelissen BJC (1989) The genetic engineering of two commercial potato cultivars for resistance to potato virus X. Bio/Technology **7**: 273-278

Johansen RH, Farnsworth B, Nelson DC, Secor GA, Gudmestad N, Orr PH (1988) Russet Norkotah: a new russet-skinned potato cultivar with wide adaptation. Am Potato J **65**: 597-604

Lawson C, Kaniewski W, Haley L, Rozman R, Newell C, Sanders P, Tumer NE (1990) Engineering resistance to mixed virus infection in a commercial potato cultivar: resistance to potato virus X and potato virus Y in transgenic Russet Burbank. Bio/Technology **8**: 127-134

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

Ooms G, Burrell MM, Karp A, Bevan M, Hille J (1987) Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. Theor Appl Genet **73**: 744-750

Park YD, Ronis DH, Boe AA, Cheng ZM (1995) Plant regeneration from leaf tissues of four North Dakota genotypes of potato (*Solanum tuberosum* L.). Am Potato J **72**: 329-338

Powell AP, Nelson RS, De B, Hoffmann N, Rogers SG, Fraley RT, Beachy RN (1986) Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. Science **232**: 738-743

Schardl CL, Byrd AD, Benzion G, Altschuler MA, Hildebrand DF, Hunt AG (1987) Design and construction of a versatile system for the express of foreign genes in plants. Gene **61**: 1-11

Sheerman S, Bevan MW (1988) A rapid transformation method for Solanum tuberosum using binary *Agrobacterium tumefaciens* vectors. Plant Cell Reports **7**: 13-16.

Shur M, Wessler S, Fedoroff N (1983) Molecular identification and isolation of the Waxy locus in Maize. Cell **35**: 225-233

Southern EM (1975) Detection of specific sequence among DNA fragments separated by gel electrophoresis. J Mol Biol **98**: 503-517

(1997년 7월 8일 접수)