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Research Article

An efficient transformation method for a potato (*Solanum tuberosum* L. var. Atlantic)

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Abstract We found that a long period of *in vitro* culture is a critical factor on the low transformation rate for a specific potato genotype, *Solanum tuberosum* L. var. Atlantic when phosphinothricin (PPT) was added to select putative transformants in a solid media. The fresh explants of the newly produced plants from a micro-tuber was able to increase the transformation rate significantly while the old explants prepared from a plant maintained for longer than 6 months *in vitro* by sub-culturing every 3 ~ 4 weeks resulted in a very low transformation frequency. However, Jowon cultivar was not so much influenced by the period of *in vitro* culture with high transformation rate (higher than 10.0%). Further research need to be explored for the reason why a particular potato genotype, Atlantic is more vulnerable than the Jowon cultivar during the regeneration stage resulting in the low transformation frequency.

Introduction

During the last fifteen years, the total cultivation area of genetically modified (GM) crops has been gradually increased and in year 2012, they were cultivated in 170,000,000 ha all over the world. Maize, soybean and cotton bearing insect-resistant gene or/and herbicide-resistant gene were the major GM crops cultivated so far (James 2013). Recently, GM potato such as amylose-free line created through RNAi technology has also been approved for the commercialization (Abdallah 2010). Moreover, potato has been considered as a good candidate crop for mass production of industrially valuable protein or pharmaceutically useful substances by using recombinant DNA technology since potato tuber tissue contains well known mechanism to accumulate proteins in protein body (Pots et al. 1999; Shewry 2003; Gerszberg et al. 2012; Ahmad et al. 2012).

However, there have been several reports that a particular genotype of potato exhibited very low transformation frequency and became a stumbling block for the development of a new GM potato. Although De Block (1988) reported a genotype-independent method was developed for potato, there have been several claims that to some extent, the efficiency of transformation is dependent on genotypes. For example, Dale and Hampson (1995) reported that out of 34 varieties, only half of them were successful for transformation by using tuber discs and some varieties were not able to transform by using either tuber discs or leaf explants. In addition, most of cultivars in Latin America, having *andigena* genetic background have been very inefficient until genotype-specific method was developed (Rodríguez et al. 2000; Trujillo et al. 2001). There are several other critical factors influencing the transformation rate including a kind of growth regulator and carbon sources such as sucrose or glucose in the regeneration media, tissues of explants and selective marker such as *nptII* and *bar* gene.

We have also experienced same problems with some potato cultivars bred in South Korea including Namseo, Chuback, Jopoong, Jasim and Jowon (Lee et al. 2003). In detail, all of them except Jowon cultivar exhibited very low transformation ratio by using either leaf or internode explants. Interestingly enough, we were able to increase the transformation efficiency up to 80% with Jowon cultivar by using available method but most of other cultivars were lower than 5.0%. Furthermore, when phosphinothricin (PPT) was added into media for the selection of transgenic plants bearing *bar* gene as a selective marker, the transformation efficiency of Jowon cultivar was decreased to lower than 20% and most of cultivars bred in South Korea showed very low transformation ratio. Since Si

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et al. (2003) reported that tuber discs explants were very efficient and fast for transformation, we also have tried but not very successful due to a very low selection pressure with PPT. Most of selected plants that have been survived in high content of PPT turned out to be false by using PCR or southern blot hybridization.

In this study, we tried to look into the effect of a long period of *in vitro* culture and glucose as a carbon source on the efficiency of regeneration and transformation with two potato cultivars, Jowon and Atlantic.

Materials and Methods

Plant Materials and Chemicals

The plant maintained in test tube for *Solanum tuberosum* L. var. Jowon and *Solanum tuberosum* L. var. Atlantic were obtained from Highland Agriculture Research Center, National Institute of Crop Science, Pyeongchang 232-955, RDA, South Korea and Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology, South Korea, individually. They were maintained *in vitro* on MS basal media (Murashige and Skoog 1962) containing 2% sucrose by sub-culturing every 3 to 4 weeks at 23±2°C and 16 hrs day and 8 hrs night condition for longer than 6 months. Explants were prepared from these plants for transformation and designated as "old explants". For the preparation of "Fresh explants", however, micro-tuber was induced from potato plants by leaving on MS basal media containing 2% sucrose with no growth regulators for longer than 7 weeks without sub-culturing. After harvesting micro-tubers, they were cultured on MS basal media containing 2% sucrose to produce a new plant and sub-cultured in a fresh media to propagate less than 2 times and used to prepare explants for transformation and designated as "Fresh explants". Chemicals including MS, Sucrose, Bacto peptone, Phosphinthricin (PPT), Carbenicillin, NAA, GA_3, Zeatin, Spectinomycin, Rifampicin, Gentamycin were purchased from Duchefa Co.

Plant Transformation

The recombinant T-DNA vector (pSIOR) was donated from Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology, deajeon, South Korea and was transformed into *Agrobacterium tumefaciens*, GV3101. Plant transformation was performed as described by Visser et al. (1989) and Kim et al. (2009). *Agrobacterium* bearing recombinant T-DNA vector was cultured on YEP media containing spectinomycin (1.0 g/L), gentamycin (25 mg/L) and rifampicin (25 mg/L) at 28°C for 2 to 3 days and adjusted the cell density to 0.6 to 07 of optical density by using spectrophotometer. Meantime the explant was pre-cultured in liquid M1 media (Table 1) for 1 day under light and was infected with *Agrobacterium* by submerging into cultured media for 15 min and then put them on whatman filter paper to remove excess *Agrobacterium* and co-cultured on M2 solid media for 2 days. And then two different regeneration methods were used to test the effect of carbon source, sucrose and glucose. The first, co-cultured explant was cultured on M3 media containing sucrose as a carbon source until transfer to rooting media. The second, the co-cultured explants was cultured on M3 media for first 4-week and then switched to M4 media containing glucose as a carbon source. The regenerated shoots were then moved to a rooting media containing no growth regulator. The chemical composition of M1 to M4 media were summarized at Table 1.

Nucleic Acid Purification, PCR and RT-PCR

Genomic DNA was extracted by CTAB method from fresh leaves by using DNeasy plant mini kit (Gene All, South Korea) as described Doyle and Doyle (1987) and Kim et al. (2009). To confirm the transgenic plant by PCR, fifty ng of genomic DNA was mixed with 5 pmol of forward primer, 250F (5'-CGGTCTGCACCATCGTCAACC-3') and reverse

Table 1 Composition of media for potato transformation used in this study

Medium	Sucrose (g/L)	Glucose (g/L)	BA (mg/L)	NAA (mg/L)	2.4-D (mg/L)	GA_3 (mg/L)	Zeatin (mg/L)	
M1	30	-	10	10	-	-	-	NH_4NO_3 (147 mg/L), $CaCl_2$ (80 mg/L)
M2	30	-	-	-	2	-	-	
M3	30	-	-	0.01	-	0.1	2	PPT (0.5 mg/L), Carbenicillin (500 mg/L)
M4	-	16	-	0.02	-	0.15	-	PPT (0.5 mg/L), Carbenicillin (500 mg/L)

primer, 251R (5'-GTCCAGCTGCCAGAAACCCAC-3') designed for *bar* gene in a reaction buffer containing Taq DNA polymerase and denatured at 94°C for 5 min and then 30 cycle of 30 sec of 94°C, 1 min of 60°C, 30 sec of 72°C was run. The reaction mixture was maintained for 10 min at 72°C and then fractionated on an agarose gel by electrophoresis. Total RNA was purified with Trizol buffer (Qiazen, Germany) as described in the provided instruction. Two hundred ng of total RNA was mixed with 5 pmol of primers designed for *bar* gene in a reaction buffer containing RT-PCR mixture (GENETBIO, South Korea) and first reaction was run at 50°C for 30 min for cDNA synthesis and then directly PCR reaction was performed in same tube as described for PCR.

Results and Discussion

In late 1980, the method for potato transformation has already been established by several research groups (Sheerman and Bevan 1988; De Block 1988; Visser et al. 1989; Wenzler et al. 1989; Beaujean et al. 1998; Mitten et al. 1990) but still some genotypes are not very efficient for transformation. In particular, it become more serious when the *bar* gene was used as a selective marker with some genotypes (De Block 1988; Lee et al. 2003; Shin and Park 2008). As summarized in Table 2, transformation rates were very fluctuated with 20 to 100% by genotypes, explants tissue, selection markers, and composition of media, in particular growth regulators. Some of *andigena* genotypes and Bitje, Berolina, Desiree and Russet Burbank exhibited better transformation rate by using glucose as a carbon source (De Block 1988; Banerjee et al. 2006) and addition of zeatin riboside and/or ascorbic acid increased the transformation rate (Trujillo et al. 2001; Si et al. 2003; Banerjee et al. 2006). De Block (1988) reported that the addition of $AgNO_3$ enhanced the transformation efficiency up to 100% with *nptII* as a selective marker while it was decreased to only 20% when the *bar* gene was used as a selective marker. BAP or BA hormone was efficient for the stem or tuber disc as explants (Stiekema et al. 1988; Beaujeon et al. 1998; Si et al. 2003).

The effect of carbon source, sucrose or glucose on the transformation efficiency

In order to prepare the explants of potato for transformation, potato plant was maintained aseptically *in vitro* and sub-cultured every 3 to 4 week intervals. As usual, we prepared explants of leaf and internodes from plants maintained for more than 6 months *in vitro* ("Old explants") and performed transformation as described in Materials and Methods. Two cultivars, Jowon and Atlantic were chosen to compare the transformation efficiency with *bar* gene. "Old explants" were pre-cultured for 1 day in M1 media under light and transferred to M2 media for co-culture with *agrobacterium* bearing recombinant T-DNA for 2 days and then divided into two conditions. First half was continuously cultured on M3 media containing 3% sucrose as a carbon source with NAA, GA_3 and zeatin and second half was cultured in M3 media for 4 weeks and then moved to M4 media containing 1.6% glucose as a carbon source and NAA, GA_3 but no zeatin. As summarized in Table 3, when the co-cultured explant of Jowon cultivar was cultured continually on M3 media containing sucrose as a carbon source, 118 to 140% of regenerated shoots were produced from leaf and stem, respectively. Out of them, 32.1 to 37.3 % were survived in media containing PPT (1.0 mg/L) and finally, 8.9 to 10.1% of them were confirmed to contain a recombinant T-DNA fragment in their genome by PCR. In addition, when the media was switched to M4 containing glucose as a carbon source after 4-week culture in M3 media, more regenerated plants were survived (44.2 to 54% of regenerated plants) on the media containing PPT (1.0 mg/L) and 16.3 to 18.4% of them were confirmed to be real transgenic plants by PCR.

Table 2 Comparison of transformation rates with the different transformation methods for several potato cultivars reported so far

Cultivars	Tissue	Selection marker	Media for shoot induction	Frequency (%)	References
Andigena	leaf	*NPTII*	glucose, NAA, GA_3, zeatin riboside	35	Banerjee et al., 2006
Bintje Berolina Desiree Russet Burbank	leaf	*NPTII* *Bar*	glucose, zeatin, GA_3, $AgNO_3$	NPTII (100) Bar (20)	De Block, 1988
Andean cultivars Diacol Capiro Parda Pastusa	leaf	*NPTII*	sucrose, Ascorbic acid, zeatin riboside, GA_3	up to 58	Trujillo et al., 2001
Bintje Desiree Kaptah Vandel	stem	*NPTII*	sucrose, BAP, NAA, GA_3	90	Beaujean et al., 1998
Bintje Desiree	tuber disc	*NPTII*	sucrose, BAP, GA_3	not specified	Stiekema et al., 1988
E-potato 3 Guannongshu-2	tuber disc	*NPTII*	sucrose, IAA, GA_3, BA, zeatin riboside	43.9 - 45.5	Si et al., 2003

Table 3 The effect of carbon sources on the transformation efficiency of potato cultivars, Jowon and Atlantic. The composition of media, M1 to M4 were described in Table 1

Variety	Media	Tissue	% of regenerated plants	% of resistant plants in PPT (1.0 mg/L)	% of transgenic plants (PCR)
Jowon	M3	leaf	118	37.3	10.1
		stem	140	32.1	8.9
	M3-M4	leaf	119	54.0	18.4
		stem	148	44.2	16.3
Atlantic	M3	leaf	12	0.0	0.0
		stem	0.0	0.0	0.0
	M3-M4	leaf	10.1	1.1	0.5
		stem	8.3	1.7	0.0

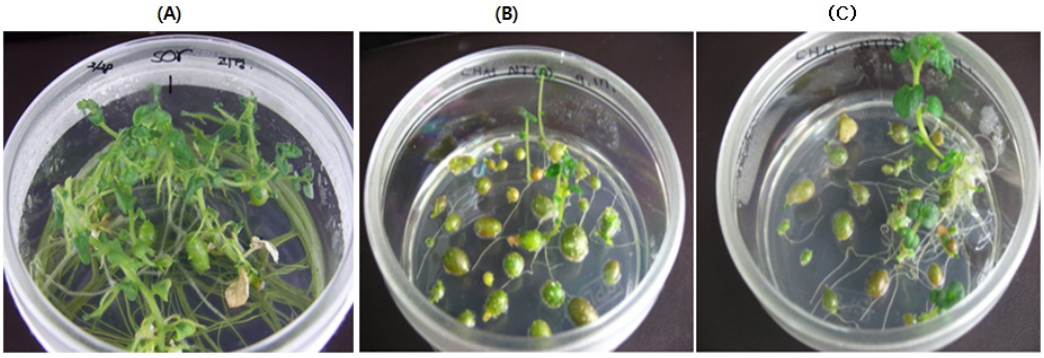

Fig. 1 The induction of mini-tuber from potato plants maintained in vitro for a long period (at least longer than 6 months). Plants were kept for 6 weeks on a solid basal MS media with 2% sucrose without sub-culturing and a new min-tuber was induced (A). After harvesting mini-tubers, they were grown on a basal MS media to produce a new fresh shoot in 2 weeks (B) and a whole plant (C) are ready to prepare a 'fresh explant' for transformation or to sub-culture for the propagation when necessary

However, for Atlantic cultivar, we could not obtain any transgenic plants confirmed by PCR using M3 media since very low number or no shoots were regenerated from both leaf and stem. By culturing M4 media after 4-week culture in M3 media, the regeneration rate from leaf explants was slightly increased (10.1%) but only 0.5% of regenerated plants were confirmed to be real transgenic plants by using PCR and no transgenic plants were able to obtain from stem explants (Table 3).

The effect of *in vitro* culture period on the regeneration and transformation rate

We then tried to observe the regeneration and transformation efficiency by using the "Fresh explants" of the newly induced plant from micro-tuber in which was harvested from the plant maintained *in vitro* for more than 6 months. As shown in Figure 1, micro-tubers were produced routinely by culturing for longer than 7 weeks in MS basal media without adding any growth hormone. After harvesting micro-tubers, they were cultured on a same media to produce a new shoot at about 3 weeks. "Fresh explants" were then prepared from a newly

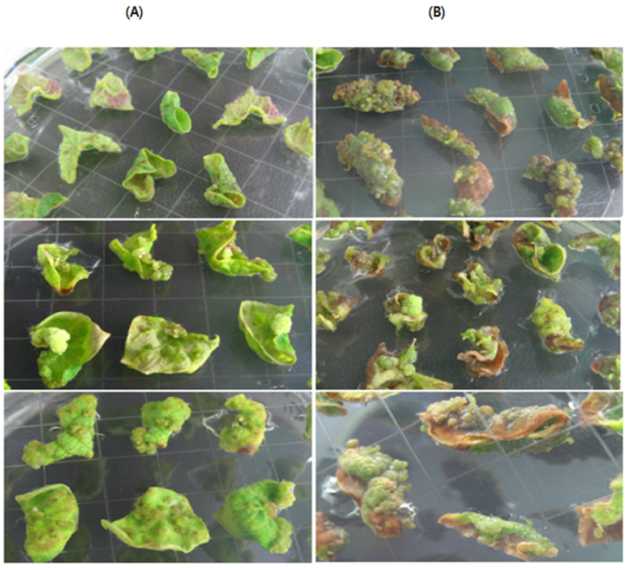

Fig. 2 Comparative experiment of callus induction rate between from 'fresh explant' (a) obtained from a plant of newly produced mini-tuber (less than 2 times sub-cultured) and from 'old explant' (b) obtain from a plant of consecutive sub-cultured more than 4 times. Photo was taken at 5 weeks after co-cultured with *agrobacterium* bearing a recombinant vector

produced plant or only 1 or 2 times sub-cultured plant if they need for propagation. The prepared "Fresh explants" were transformed with recombinant T-DNA by using same method for pre-culture in M1 media and co-culture in M2 media but after culturing in M3 for 4 weeks and then switched to M4 media. Interestingly enough, "the Fresh explants" exhibited very active induction of callus as well as shoot. Moreover, shoots were very healthy with a bright green color while "Old explants" prepared from Atlantic cultivar turned out to be brown and died at this stage (Fig. 2, Fig. 3). Such a clear difference was not observed with Jowon cultivar showing that even "Old explants" showed very active callus and shoot induction and stayed healthy throughout regeneration stage. As summarized in Table 4, for Atlantic cultivar, "Fresh leaf or stem explants" showed high frequency of regenerated plants (71.7 for leaf and 20.5% for stem) and 11.2% (leaf) and 8.3% (stem) out of them were survived on the media containing PPT. Finally 4.3% (leaf) and 2.5% (stem) out of them were confirmed to contain the recombinant T-DNA fragment in their genome by PCR and RT-PCR (Fig. 4). For Jowon cultivar, we also found a significant effect of the "fresh leaf explants" by increasing 7.1% compared to that of "old explants" but for stem explants, the effect was not so much (only 1.5% increased).

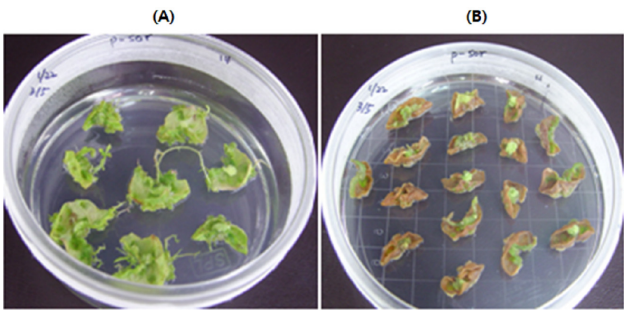

Fig. 3 Comparative experiment of shoot induction rate between from 'fresh explants' (a) and from 'old explants'. Photo was taken at 3 - 4 weeks after transferred on shoot induction media

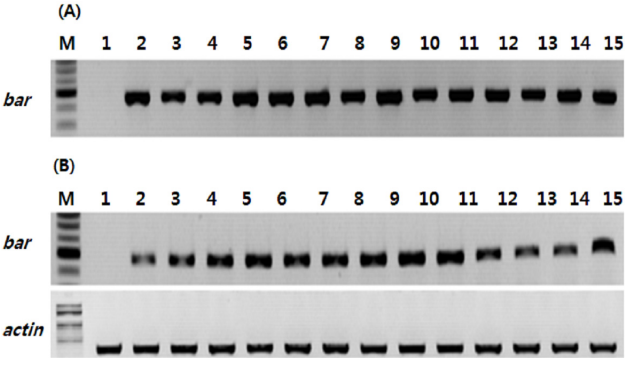

Fig. 4 Results of PCR and RT-PCR analyses for the confirmation of transgenic potato plants. Genomic DNA and total RNA were purified from a negative control, Atlantic cultivar, a parental line (lane 1) and candidate transgenic plants (lane 2 to 15) and the PCR or RT-PCR reaction was performed with a primer set designed for *bar* gene or *actin* gene. The PCR reaction product (A) and RT-PCR products (B) were fractionated on agarose gel, respectively

In this report, we found that the short period of *in vitro* culture is critical to increase the transformation rate of some potato cultivars. Since potato is vegetative reproduction plant and easily propagated *in vitro* aseptically, explants is normally prepared from the plant maintained *in vitro* for transformation. However, we realized that a long period of *in vitro* culture cause a serious problem during the regeneration for Atlantic cultivar in which is very popular and cultivated in the largest area of China. At present, we do not have any clear idea for the reason why the Jowon cultivar was not so much influenced by a long period of *in vitro* culture. One can assume that a somaclonal variation during a long period of the *in vitro* culture may be involved in the low frequency of regeneration of some potato genotypes. Somaclonal variation has been detected in potato plants regenerated from petiole, leaf (Austin and Cassells 1983) and protopalsts (Shepard et al. 1980). It has been suggested that a minimum time *in vitro* culture is

Table 4 The effect of sub-culture peroid of potato explants on the regeneration and transformation frequency

Cultivar	Sub-culture	Tissue	% of Regenerated plants	% of resistant plants in PPT (1.0mg/L)	% of transgenic plants (PCR)
Jowon	Fresh explants[a]	leaf	126	65.8	25.5
		stem	132	51.2	17.8
	Old explants[b]	leaf	138	54.0	18.4
		stem	52	44.2	16.3
Atlantic	Fresh explants	leaf	71.7	11.2	4.3
		stem	20.5	8.3	2.5
	Old explants	leaf	9.3	1.5	0.5
		stem	8.0	2.0	0.0

[a]prepared from a plant induced from micro-tuber or maintained less than 1 - 2 months (1-2 times sub-cultured).
[b]prepared from a plant maintained longer than 6 months (more than 6 times sub-cultured)

necessary to avoid undesired somaclonal variation for the development of transgenic crop (Birch 1997).

In summary, our preliminary results in the present study indicate that a long period of *in vitro* culture to provide explants aseptically for the transformation of vegetative propagating crops including potato may cause a somaclonal variation resulting in the low transformation rate and it is highly genotype dependent.

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