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Protocols for *Agrobacterium*-mediated Transformation of Potato

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ABSTRACT

This review presents a collection of 27 studies dedicated to *Agrobacterium*-mediated transformation of potato. Studies were selected in order to present the gradual improvement of transformation techniques from early research to routine techniques used in many laboratories in everyday work. Some of the protocols were selected on the basis of their popularity and application in certain lines of transgene research. Apart from the more widely used *A. tumefaciens* there are protocols adjusted for *A. rhizogenes*-mediated transformation. Protocols for different explant types (shoots, leaves, tubers) are represented just as those elaborated specifically for the most important commercial cultivars like 'Désirée', 'Bintje', 'Shepody', 'Kennebec' and 'Russet Burbank'. Emphasis in each protocol is placed on the growth response of explants exposed to various plant growth regulators and other substances and conditions which support shoot regeneration. Attention was also paid to protocols and treatments which may suppress the regeneration of aberrant, off-type plants.

Keywords: co-cultivation, excised root cultures, inoculation, *in vitro*, shoot regeneration

Abbreviations: 2,4-D, dichlorophenoxyacetic acid; **Augm**, augmentin; **AS**, adenine sulphate; **BA**, benzyl adenine; **BS**, bacterial suspension; **Carb**, carbenicillin; **Cef**, cefotaxime; **CIM**, callus induction medium; **Claf**, claforan; **CH**, casein hydrolyzate; **FCL**, feeder cell layer (FC layer); **GA_3**, gibberellic acid; **Hygro**, hygromycin; **IAA**, indol acetic acid; **IAA aspartic acid**, conjugate form; **Kin**, kinetin; **Km**, kanamycin; **LM**, liquid medium; **MS**, Murashige and Skoog (1962); **NAA**, naphthyleneacetic acid; **PGR**, plant growth regulators; **Ri-transformed**, *Agrobacterium rhizogenes* transformed; **SIM**, shoot induction medium; **Tim**, timentin; **Vanc**, vancomycin; **Zea**, zeatin; **ZR**, zeatin riboside

CONTENTS

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Invited Review

INTRODUCTION

Genetic engineering of potato is currently one of the most propulsive research fields in potato biotechnology with numerous contributions both in basic and applied research. Several factors contribute to such a high interest. Potato is an important, multipurpose crop species, used for food, feed and industrial processing. It is positioned as the fourth most important crop species in the world. Commercial potato which we mostly cultivate comprises a group of highly heterozygous, tetraploid cultivars in which improvement by conventional breeding techniques is cumbersome and difficult. Plant biotechnology techniques amply demonstrated their potential in potato breeding. *Agrobacterium*-mediated transformation which we will present here enables accurate introduction of single, desired traits in already established cultivars. Introduction of desired traits can be used also in basic research. Overexpression or silencing of certain genes provides a tool of utmost importance in studies of the pathways of plant metabolism.

For those entering the field of potato transformation and genetic engineering the most difficult obstacle is the existence of many previously published papers in which it is difficult to find the leading points. Investigators usually stick to protocols developed by other research teams used in studies which they continue. Sometimes, they are not aware of the existence of other protocols, which may be even better suited for their needs.

We recently surveyed more than 500 journal articles covering various topics of transgene potato research. It enabled us to prepare this review compiling data for 27 most frequently used protocols for *Agrobacterium*-mediated transformation of potato. Some of the presented protocols are still very popular in certain research lines while the others are only of historical importance. For newcomers it is usually difficult to understand the true importance of various steps or operations comprising a protocol. Some of the steps once considered highly important have been abandoned through time and replaced with other equally important ones. Some steps were showed to be unnecessary (redundant) or even detrimental for cultures.

Transformation success in potato is highly dependent on the genotype and that is the main reason for the existence of many different protocols. The effect of bacterial strain and type of vectors and constructs is of far less importance since we can choose and change them according to our needs.

This presentation is therefore focused mostly at the employment of *in vitro* culture techniques with emphasis on (1) choice of explants, (2) bacterial inoculation, and (3) explant growth responses with shoot and plantlet regeneration as the final goals.

PROTOCOLS – GENERAL CONSIDERATIONS

Before we advance to the presentation and analysis of individual protocols it is useful to summarize in general factors, conditions and operations comprising potato transformation protocols. Numbers in parenthesis correspond to the number under which protocols are listed in **Tables 1A** and **1B**.

Mineral salt formulation

All protocols with only few well marked exceptions use the Murashige and Skoog (1962) MS medium formulation. In most protocols MS medium is supplemented with 2-3% sucrose. Carbohydrate composition is stated only if it differs from this standard value.

Potato cultivars and genotypes

The most frequently investigated potato genotype was cv. 'Désirée' (5, 6, 7, 8, 9, 10, 15, 17, 18, and 21). Well elaborated procedures were also established for cultivars 'Bintje' (7, 10, 12, 16, and 21), 'Russet Burbank' (3, 7, 8, and 9), 'Kennebec' (16) and 'Shepody' (26).

Bacterial strain

Agrobacterium tumefaciens-mediated transformation is today considered as the main and routine technique for potato transformation. LBA 4404 is the strain most frequently used in combination with different constructs introduced by binary vectors. *A. rhizogenes* can be also used for gene transfer, offering uniform transgene progeny.

Explant source

The most common explant sources are leaves, shoots and tubers. They can all support efficient production of transgenic plants. However, most of the studies were done with leaf explants.

In a summary presented in **Tables 1A** and **1B**, protocols are grouped according to the utilized explants.

Leaves are usually cut into smaller strips or squares. They can be also prepared as discs using a corkscrew borer. Some procedures use whole leaves just detached from the petiole while others use the central parts of the lamina after its apical and basal parts have been removed. Wounds made by explant preparation are sufficient although some procedures insist on additional cuts made across the main leaf veins. The adaxial or abaxial sides of the leaves are usually in contact with medium. According to Dietze et *al*. (1995) the youngest leaves at the apical meristem and the oldest leaves at the base of the plants will not give rise to transformed shoots at a good frequency. Cutting off the leaf base and making two parallel 4-5 mm long incisions over the leaf midrib and insertion of the explant in the opposite position is also recommended.

Shoot explants are prepared as nodal cuttings or as internodes; the latter avoids the presence of axillary buds. Shoot explants can be inserted in a normal or inverted position, intact or cut longitudinally in half. The traditional, oldest inoculation method employs wounding of shoot ex-

Table 1A Summary of potato transformation protocols. Explant inoculation and shoot regeneration.

Authors, year	Cultivar, genotype or species	Explant source	Explant preculture (prior to inoculation)	Inoculation conditions and duration (days)	Callus induction medium CIM	Shoot induction medium SIM	Root induction medium RIM
LEAF EXPLANTS							
Ann *et al*. 1986	'Russet Burbank', ADX262-9	leaves and shoots	no preculture	Liq. MS + BS 2 days	BA 0.5 2,4-D 2.0 glutamine 15	BA 0.5 glutamine 15	not stated
Shahin and Simpson 1986	NDD-277-2	leaves	BA 0.4 NAA 1.2 callus produced 8 days	Liq. MS + BS 2 days	BAA 0.45 NAA 0.18 AS 40.0 CH 50.0 3 weeks	Shahin (1984) 3 weeks	MS + 0.17 GA_3 + Cef 50
De Block 1988	'Bintje', 'Désirée', 'Berolina', 'Russet B'	leaves	no preculture	Liq. MS + BS 2 days	Zea 1.0 NAA 0.1 AS 40 1-2 weeks	Zea 1 AS 40 glutamine 200 glucose 2% mannitol 2% buffers, $AgNO_3$	PGR-free Gamborg B5 + 2% sucrose + antibiotics
Rocha Sosa *et al*. 1989, or Keil *et al*. 1989	'Berolina' 'Désirée'	leaf disks	no preculture	Liq. MS +BS in darkness 2 days	glucose 1.6% ZR 2.0 NAA 0.02 GA_3 0.02 30-40 days	-	PGR-free MS + 2% sucrose + antibiotics
Tavazzaa *et al*. 1988	'Désirée'	leaf explant	'Désirée' FC layers + Kin 0.25 + 2,4-D 5.0 1 day	BS 1-2 min followed by 'Désirée' FCL Kin 0.25 + 2,4-D 5.0 2 days	BA 1.0 IAA 1.0 GA_3 10.0 antibiotics 3-4 weeks	-	PGR-free 1% sucrose
Wenzler *et al*. 1989	FL1607, 'Désirée', 'Russet Burbank', 'Superior'	leaf strips	CIM, 4 days	BS 10 min followed by CIM, 4 days	BA 2.25 NAA 0,2 GA_3 10.0 antibiotics 12 days	BA 2.25 GA_3 10.0 antibiotics 4 weeks	not stated
Edwards *et al*. 1991	'Désirée'	leaf squares	no preculture	BS 30 min followed by CIM, 2 days	Zea 0.5 2,4-D 2.0 Augm 100 1 day followed by CIM 4 days	Zea 0.5 GA_3 2.0 antibiotics 6 weeks	PGR-free MS + sucrose 2%
Dietze *et al*. 1995	'Désirée'	leaves with cuts above midrib	no preculture	LMS + BS 2 days in darkness	glucose 1.6% BA 0.1 NAA 5.0 7 days	ZR 2.0 NAA 0.02 GA_3 0.02 2 subcultures	MS + sucrose 2% Claf 250
Trujilo *et al*. 2001	'Diacol Capiro', 'Parda Pastusa'	leaves	liq. PGR-free MS 4 days	BS 10 min followed by liq. MS + sucrose 1% for 1 day followed by CIM 3 days	ZR 3.0 GA_3 1.0 CH 0.05% 3 days 5-9 weeks	-	PGR-free MS antibiotics
Barell 2002	'Iwa'	leaves	no preculture	BS 30 sec followed by BA 2.0 NAA 0.2 ascorb. acid 40.0 CH 500.0 2 days	BA 2.0 NAA 0.2 ascorb.acid 40.0 CH. 500.0 Tim 200 5 days followed by select. markers 2-6 weeks	Zea 1.0 GA_3 5.0 sucrose 0.5% ascorb.acid 40.0 CH 500.0 antibiotics + sel. agents	PGR-free MS + antibiotics
Gustafson *et al*. 2006**	'Shepody'	leaf segments, internode segments	no preculture	BS + aceto syringone 72.5 2 min followed by CIM, 2 days	Zea 1.0 NAA 0.1 antibiotics 2 x 10 days	Zea 1.0 antibiotics 1-2 weeks	PGR-free + antibiotics
Banerjee *et al*. 2006	*Solanum andigena*	leaves	no preculture	PGR-free MS + BS, 2 days	BA 0.1 NAA 5.0 glucose 1.6% 7-8 days	ZR 2.2 NAA 0.02 GA_3 0.15 glucose 1.6% subcultured every 10 days	PGR-free MS + sucrose 2% antibiotics

Table 1A (Cont.)

Authors, year	Cultivar, genotype or species	Explant source	Explant preculture (prior to inoculation)	Inoculation conditions and duration (days)	Callus induction medium CIM	Shoot induction medium SIM	Root induction medium RIM
SHOOT AND STEM EXPLANTS							
Visser *et al*. 1989b	7322, 86.040	stem, leaf	liquid MS BAP 10.0 NAA 10.0 overnight	BS 15 min followed by CIM, 2 days	BA 2.25 IAA 0.017 sucrose 0.1% mannitol 0.4% + antibiotics 5-7 days	sucrose 1.5%. BA 2.25 GA_3 5.0 antibiotics 4-6 weeks	PGR-free MS + sucrose 3%
Newell 1991	'Ruset Burbank'	stem internodes, 6mm long	no preculture	cut smearing, Tobacco FC layers + CIM, 2 days	BA 3.0 NAA 0.01 antibiotics 4 weeks	GA_3 0.3 antibiotics 2 month subc	PGR-free MS + antibiotics
Beaujean *et al*. 1998	'Désirée' 'Bintje', 'Kaptah Vandel'	internode explants 4-6 mm cut lengthwise	no preculture	BS 30 min followed by CIM, 3 days	ZR + 0.8 2,4-D 2.0 9 days	ZR 0.8 GA_3 2.0 antibiotics	IAA 0.1 antibiotics
Ducreux *et al*. 2005	*Solanum phureja* DB337/37 'Mayan Gold'	internodes, leaves and petioles	Explants collected in LM	LM + BS 5-10 min followed by CIM, 2 days	ZR 2.5 NAA 0.2 Cef 12 days	ZR 2.5 NAA 0.2 GA_3 0.02 both antibiotics 3 x 14 days	PGR-free + sucrose 3% + antibiotics
Heeres *et al*. 2006	16 cultivars	stem internodes	LM 1 day	BS + 2 days	ZR 1.0 Zea 0.5 2,4- D 2.0 5 days	- Zea 0.5 GA_3 2.0 6 weeks	PGR-free + sucrose 2% + antibiotics
TUBER EXPLANTS							
Stiekema *et al*. 1988	'Bintje' 'Désirée'	tuber slices 3mm	no preculture	'Bintje' FCL + CIM, 3 days	Zea 1.0 NAA 0.01 2 weeks	BA 0.25 GA_3 0.1 2-3 weeks	IAA 0.1 antibiotics
Sheerman and Bevan 1988	'Désirée' and 6 more cultivars	tuber slices 1-2 mm	no preculture	MS + BS 20 min followed by Tobacco FCL + CIM, 2 days	ZR 1.75 IAA aspartate 0.9 4-6 weeks	-	PGR-free + antibiotics
Kumar *et al*. 1995	wild species	microtuber slices	no preculture	LM + BS 30 min followed by CIM, 2 days	ZR 1.8 IAA-aspartate 0.9 antibiotics subcultured every 2 weeks	kin 0.1 GA_3 0.2 1.5% sucrose antibiotics	not stated
***A. rhizogenes* mediated transformation**							
De Vries Uijtewaal 1988	8 haploid, 2 diploid lines	stem internodes	no preculture	LM 60 min	-	-	MS + sucrose 5% Cef 200 3-4 weeks
Hänish ten Cate *et al*. 1988	'Bintje' 'Désirée'	leaf segments tuber discs	Explants placed on 1.5% agar	explants smeared with 0.1 ml BS	induction from roots followed by Zea 2.25 2,4-D 12.0 8 weeks	induction from hairy root induced callus followed by Zea 2.25 2,4-D 0.12 8 weeks	MS + Cef 200
Ottaviani *et al*. 1990	'Bintje'	various	Explants placed on 1.5% agar	explants smeared with 0.1 ml BS	induction from hairy roots followed by ZR 3.0 2,4-D 0.05-0.1	induction from hairy root induced callus followed by ZR 3.0 GA_3 1.0	MS + Cef 300
Visser *et al*. 1989a	cultivars of various ploidy levels	stem segments 2-3 mm	no preculture	15 min + 2 days more on antibiotic free medium	PGR-free MS + Cef followed by Zea 2.0 2,4-D 0.12	BA 2.25 GA_3 10.0 antibiotics	PGR-free MS + sucrose 3% + Cef
Dobigny 1995	'Fanette'	internodes	NAA up to 5.0	cut end smeared with bacteria	spontaneous shoot regeneration	-	MS + Cef 500

Concentrations of all PGRs and other media constituents are expressed in mg/l. Only sucrose and other carbohydrates are expressed as w/v percentages

Abbreviations

2,4-D, dichlorophenoxyacetic acid; **Augm**, augmentin; **AS**, adenine sulphate; **BA**, benzyl adenine; **BS**, bacterial suspension; **Carb**, carbenicillin; **Cef**, cefotaxime; **CIM**, callus induction medium; **Claf**, claforan; **CH**, casein hydrolyzate; **FCL**, feeder cell layer (FC layer); **GA_3**, gibberellic acid; **Hygro**, hygromycin; **IAA**, indol acetic acid; **IAA aspartic acid**, conjugate form; **Kin**, kinetin; **Km**, kanamycin; **LM**, liquid medium; **Liq.**, liquid; **MS**, Murashige and Skoog (1962); **NAA**, naphthyleneacetic acid; **PGR**, plant growth regulators; **SIM**, shoot induction medium; **Tim**, timentin; **Vanc**, vancomycin; **Zea**, zeatin; **ZR**, zeatin riboside

Table 1B Summary of potato transformation protocols. Bacterial strains, plasmids genes and bacterial inactivation.

Authors, year	Cultivar, genotype or species	Bacterial strain plasmids and genes	Explant source	Antibiotics (highest concentrations, mg/l)
LEAF EXPLANTS				
Ann *et al*. 1986	'Russet Burbank', ADX262-9	pGA472 pTiBo542 pAL4404 *nptII*	leaves and shoots	Km 200 Carb 500
Shahin and Simpson 1986	NDD-277-2	LBA 4404 pARC8 *nptII*	leaves	Cef 250 Km 50
de Block 1988	'Bintje', 'Désirée', 'Berolina', 'Russet Burbank'	C58C1/ pGV2260, pMP90, pGSFR1161 *nptII*, *bar*	leaves	Carb 1000 Cef 250
Rocha Sosa *et al*. 1989, or Keil *et al*. 1989	'Berolina' 'Désirée'	pGV2260 LBA4404 patatin I class gene in pBIN19 chimeric patatin I-GUS gene	leaf disks	Claf 500 Km 50
Tavazza *et al*. 1988	'Désirée'	A 136 LBA4404 *nptII*	leaf explant	Vanc 200 Cef 200 Km 100
Wenzler *et al*. 1989	FL1607, 'Désirée', 'Russet Burbank', 'Superior'	LBA4404 pPS20A-G (patatin I class) chimeric patatin I-GUS gene	leaf strips	Carb 500 Km 50
Edwards *et al*. 1991	'Désirée'	A.t.GV3101 pGV3850 pDUB 126a,130,133 CaMV35S-LecA (pea lectin) TssRUBISCO-LecA	leaf squares	Augm 100 Km 100
Dietze *et al*. 1995	'Désirée'	not stated	leaves with cuts above midrib	Claf 250 Km 50 or Hygro 1.0
Trujilo *et al*. 2001	'Diacol Capiro','Parda Pastusa'	LBA 4404 pBi 121 *nptII*, *bar*	leaves	Carb 500 Km 100
Barell 2002	'Iwa'	EHA 105 pMOA 1-5 *nptII*, ble *hpt*, *dhfr, bar*)	leaves	timentin 200 + 5 different selective agents (**Table 3**)
Gustafson *et al*. 2006**	'Shepody'	LBA 4404 pSOL6	leaf segments, internode segments	Cef 300 Km 100
Banerjee *et al*. 2006	*Solanum andigena*	GV2260 pCB201 cDNA of StBEL5	leaves	Cef 250 Km 50
SHOOT AND STEM EXPLANTS				
Visser *et al*. 1989b	7322, 86.040	LBA 4404 pVU1011 *nptII*	stem, leaf	Cef 200 Km 50
Newell 1991	'Ruset Burbank'	pMON9809 pMON9898 CaMV35S-PVX/PVY virus coat protein genes; *nptII*	stem internodes, 6mm long	Carb 500 Km 100
Beaujean *et al*. 1998	'Désirée' 'Bintje', 'Kaptah Vandel'	C58C1Rif1 pGS Gluc1 *nptII, bar*	internode explants 4-6 mm cut lengthwise	Cef 300 Km 125
Ducreux *et al*. 2005	*Solanum phureja* DB337/37 'Mayan Gold'	LBA4404 crtb phytoene synthase gene	internodes, leaves and petioles	Cef 500 Km 50
Heeres *et al*. 2006	16 cultivars	LBA 4404 pKGBA50 Visser 1991 LBA 4404 pKGBA50 Edwards *et al*. 1991	stem internodes	Cef 200 Km 100
TUBER EXPLANTS				
Stiekema *et al*. 1988	'Bintje' 'Désirée'	LBA 4404 pRAL 4404 pBI 121 *nptII*; GUS	tuber slices 3mm	Cef 200 Km 50-100
Sheerman and Bevan 1988	'Désirée' and 6 more cultivars	LBA 4404pBin6 *nptII*	tuber slices 1-2 mm	Carb. 500 Km 100
Kumar *et al*. 1995	wild species	C58 pGV3850: pKU2 *nptII*	microtuber slices	Cef 250 Km 150

Table 1B (Cont.)

Authors, year	Cultivar, genotype or species	Bacterial strain plasmids and genes	Explant source	Antibiotics (highest concentrations, mg/l)
***A. rhizogenes* mediated transformation**				
De Vries Uijtewaal, 1988	8 haploid, 2 diploid lines	A.t.LBA 1020/pRi1855::Tn5) LBA 9402/ pRi 1855 LBA 9365/ pRi 8196	stem internodes	Cef 200
Hänish ten Cate *et al*. 1988	'Bintje' 'Désirée'	LBA 9402 AR 15834	leaf segments tuber discs	Cef 200
Ottaviani *et al*. 1990	'Bintje'	15834 1855	various	Cef 300
Visser *et al*. 1989a	cultivars of various ploidy level	A.tLBA4404 A.r.LBA1334 A.t.AM8706 A.r. AM8703 pBI121 pRK2013 *nptII*; GUS	stem segments 2-3 mm	Cef 200 Km 50
Dobigny 1995	'Fanette'	15834 2659 2659 GUS 8196GUS	internodes	Cef 500

Concentrations of all PGRs and other media constituents are expressed in mg/l. Only sucrose and other carbohydrates are expressed as w/v percentages
Abbreviations
2,4-D, dichlorophenoxyacetic acid; **Augm**, augmentin; **AS**, adenine sulphate; **BA**, benzyl adenine; **BS**, bacterial suspension; **Carb**, carbenicillin; **Cef**, cefotaxime; **CIM**, callus induction medium; **Claf**, claforan; **CH**, casein hydrolyzate; **FCL**, feeder cell layer (FC layer); **GA_3**, gibberellic acid; **Hygro**, hygromycin; **IAA**, indol acetic acid; **IAA aspartic acid**, conjugate form; **Kin**, kinetin; **Km**, kanamycin; **LM**, liquid medium; **Liq.**, liquid; **MS**, Murashige and Skoog (1962); **NAA**, naphthyleneacetic acid; **PGR**, plant growth regulators; **SIM**, shoot induction medium; **Tim**, timentin; **Vanc**, vancomycin; **Zea**, zeatin; **ZR**, zeatin riboside

plants using a sterile needle, toothpick, forceps or similar.

Tuber explants are usually 1-3 mm thick slices excised from tubers or microtubers. Older tubers which spent considerable time in storage should be avoided as explant donors. Explant may contain only inner tissues (perimedulary zone). Tuber explants containing surface tissue layers can produce non-transformed escapes (Ishida *et al*. 1989).

Explant preculture

It is sometimes assumed that excision is a stress for detached plant organs and tissues, requiring a period of recovery prior to the bacterial inoculation. Preculture can be done briefly just by collecting explants in liquid medium prior to inoculation (24). Actually wounding releases compounds actively attracting bacteria. Thus protocol 26 recommends the addition of acetosyringone (AS).

Preculture usually lasts 1, 2 or up to 4 days (8, 9, 15, and 22). There is a protocol (4) in which preculture lasted 8 days enabling sufficient time for the explants to produce callus. Preculture media usually have the same plant growth regulator (PGR) composition and balance as media used later for callus and/or shoot induction.

Feeder cell layers (FCLs) are treatments in which the medium surface is covered with a thin layer of cell suspension cultures. It is supposed that suspension cells can help in the nutrition of explants. The FCL can be prepared from tobacco (5, 16) or from potato cell suspensions (6, 9). FCLs were also used after inoculation with the intention to provide favorable conditions for T-DNA transfer from bacteria to plant cells. The use of FCLs for both preculture and inoculation has been abandoned as unnecessary in later, recently developed protocols.

Inoculation

Inoculation is a very important stage strongly affecting the overall success of the transformation procedure. Production of viable bacteria, ready to perform transformation is a prerequisite which shall not be discussed. If the initial transformation attempts in a study are negative, preparation of bacterial suspension and its effect on transformation efficiency are first to be checked and repeated as soon as possible.

In the first studies done by Ooms *et al*. (1983, 1985) inoculation was done in the traditional way by explant wounding. A sharp needle, toothpick or some other previously sterilized instrument was used to make incision in potato shoot explants. Some older protocols use the tuber disc technique by Annand and Heberlein (1977). Later it was confirmed that wounding is not necessary since explant excision creates a sufficient cut surface on which calli and regenerated shoots will appear. Additional incisions across major leaf veins have been recommended by (21).

Thus the bacterial suspension (BS) of adequate concentration, or liquid medium (LM) mixed with BS is allowed to get in contact with the explants positioned in agar solidified media or floating on top of liquid medium.

Inoculation, expressed as contact of plant explant with bacteria is usually short, lasting from 1-2 min (8, 23, and 26) to moderate 10-30 min in most procedures. Prolonged contact of bacteria and explants lasting for 2 days as recommended in older protocols (3, 4, 7, and 15) recently appeared again (25).

In most protocols explants are in direct contact with bacteria for some 10-30 min. Then they are blotted dry with sterile filter paper if not rinsed with autoclaved water or liquid medium. Explants are then placed on media on which bacterial cells will be left undisturbed for the next two days to perform the T-DNA transfer. Media is usually a callus induction or combined, callus + shoot induction type lacking only antibiotics.

Light

Some protocols (15, 18, 22, 23) recommend darkness or decreased irradiance through the inoculation stage. This can also be interpreted as a measure restricting the expected, normal growth of explant tissues.

Temperature

There are reports that even moderate temperatures in the growth room (above 19°C) may have negative effect on the transformation success (8). Most of the protocols are however performed at standard growth room temperatures, like 24°C (6) and similar values.

Inactivation of bacteria

Inoculation ends with the addition of antibiotics. Inactivation of bacteria is usually done with 500-1000 mg/l Carb (carbenicillin) or 250-300 mg/l Cef (cefotaxime) or Claf (claforan). Other antibiotics used to control bacteria include Tim (timentin) at 200 mg/l (23) or Vanc (vancomycin) (8).

Potato is highly resistant to all mentioned antibiotics easily surviving these high concentrations.

Selection of transformants

Kanamycin (Km) is unambiguously the most efficient selective agent for potato. Resistance to the Km presence is a positive proof that tissue or plant has been transformed and expresses the *npt*II gene present in the T-DNA of the bacterial vector. Km is usually applied at concentration 50-100 mg/l. Km is actually highly toxic to normal, non-transformed cells and kills or inactivates them at rather low concentrations. Thus after some 2-3 weeks on Km-supplemented media, explants are a mixture of necrotic, dying, non-transformed cells and healthy, transformed ones. Non-transformed roots are highly sensitive to the presence of Km and therefore rooting medium as a rule is supplemented with Km.

To increase the transformation efficiency it is recommended that the application of selective agent should be delayed up to 5 days enabling cell division in explants (23).

Other selective markers like metotrexat (*dhfr*), hygromycin (*hpt*), phleomycin (*ble*), phosphinothricin (*bar*) are far less efficient in tissue transformation in comparison to Km (*npt*II) (23).

Carbohydrates

Carbohydrates in the form of sucrose required for growth of plant tissues are actually not necessary for the growth of bacteria. To support bacterial growth and transformation some protocols recommend replacing sucrose with glucose (7, 15, 18, 27) and/or mannitol (7, 14), or decrease of sucrose concentration (8, 12, 14, 19 and 22). High sucrose and glucose concentrations were shown to trigger expression of transferred patatin genes (15).

Removal of ethylene

Potato tissues can generate considerable amount of ethylene. In some culture vessels with inadequate ventilation local accumulation of ethylene may appear, affecting the growth habit of cultures and transformation efficiency. Ethylene can be efficiently removed by the addition of $AgNO_3$ alone or in combination with sodium thiosulphate (7). Use of silver thiosulphate for improved growth of potato shoot cultures has been also recommended by Hulme *et al*. (1992). Later studies showed that addition of anti-ethylene compounds is not necessary in well aerated culture vessels stopped with cotton wool plugs (Chanemougasoundharam *et al*. 2004).

Regeneration stages

Following inoculation explants are placed on media expected to enable regeneration of shoots from transformed cells. Shoot regeneration can be *direct* based on a single regeneration medium or *indirect* requiring two or more regeneration media.

Direct regeneration - single step (stage) protocols are focused on avoiding the production of callus tissue at any cost. Avoiding callus tissue as a stage in the protocol is supposed to prevent somaclonal variation enabling production of normal, non-aberrant plants. This approach was made possible since the shoot regeneration of various potato explant types has been well elaborated in earlier studies (**Table 2**). Single-stage protocols, although simple, are highly efficient. Typical single-stage transformation protocols are those of Tavazza *et al*. (1988), Sheerman and Bevan (1988), Rocha-Sosa *et al*. (1989), Keil *et al*. (1989) and Trujilo *et al*. (2001).

Indirect regeneration - two step (stage) protocols use media with at least two different PGR balances. The first medium usually favors production of callus while the second favors regeneration or development of shoots. Dominant PGRs in the first media are auxins which in the second media are absent and replaced with GA_3 or are present at a very low concentration.

Indirect shoot regeneration protocols do not necessarily produce more callus than direct regeneration protocols. The presence of Km efficiently prevents development of non-transformed callus inducing also necrosis in the non-transformed explant cells. As a consequence somaclonal variation and formation of aberrant plants is low as in the direct regeneration approach.

It is apparent that both direct and indirect transformation strategies enable successful transformation of potato. It also seems that none of the numerous PGR combinations and balances is superior to the others. Among auxins it is not possible to indicate one of them as being superior to others. In the case of cytokinins, the situation is different since some observations (21, 23) favor zeatin (Zea)-type cytokinins and ZR to BA (6-benzyl adenine).

CLASSIFICATION OF REGENERATION MEDIA

In the section dedicated to protocols the abbreviation CIM standing for callus induction medium denotes the first regeneration medium containing antibiotics preventing further bacterial growth. The main component of CIM media are auxins. The second regeneration medium, designated SIM for shoot induction medium, is applied after CIM. It is usually an auxin-free medium containing cytokinins and GA_3 (gibberellic acid). In single-stage protocols there is only one regeneration medium referred to as CIM. It should be noted that the protocol of de Block (1988) has three regeneration

Table 2 Shoot regeneration from various potato explant types and callus tissues.

Direct regeneration - single stage techniques		
Roest and Bokelmann 1977	BA 1.0 + IAA 1.0 + GA_3 10.0	Regeneration from plant rachis
Binding *et al*. 1978	Kin 3.2 + IAA 0.87; BA 0.56 + NAA 0.93	Regeneration from protoplast derived calli
Jarret *et al*. 1980a	BA 3.0 + NAA 0.03 + GA_3 0.3	Regeneration from callus
Kikuta and Okazawa 1984	Zea 0.5 + IAA 0.1	Tuber explants
Sheerman and Bevan 1988	ZR 1.75 + IAA-aspartic acid 0.9	Direct regeneration from tuber disc explant
Park *et al*. 1995	ZR 4.0 + IAA 3.5	Direct regeneration from leaf explants
Esna-Ashari and Villiers 1998	BA 1.0	Direct regeneration from tuber explants,
Yee *et al*. 2001	BA 3.0 + IAA 0.5 + GA_3 1.0	Direct regeneration from petioles
Indirect regeneration - 2 stage techniques		
Webb *et al*. 1983	BA 2.25 + NAA 0.18 → BA 2.25 + GA_3 10.0	Leaf explants
Wheeler *et al*. 1985	Zea 0.5 + 2,4-D 0.5 → Zea 0.5 + GA_3 5.0	Various explant types, and the recommended No. 12 medium
No. 12 medium		
Hulme *et al*. 1992	BA 2.25 + IAA 0.018 → BA 2.25 + GA_3 5.0	Comparison of various methods (Keil *et al*. 1989; Wenzler *et al*. 1989; de Blok 1988)
Yadav and Sticklen 1995	ZR 0.8 + 2,4-D 2,0→ ZR 0.8 + GA_3 2.0	Leaf explants
Gustafson *et al*. 2006	Zea 1.0 + NAA 1.0 → Zea 1.0	Leaf explants

Abbreviations: **BA**, 6-benzyladenine; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **GA_3**, gibberelic acid; **IAA**, indole-3-acetic acid; **Kin**, kinetin; **NAA**, naphthyleneacetic acid; **Zea**, zeatin; **ZR**, zeatin riboside.

media designated as CIM, SIM1 and SIM2.

Rooting

Shoots of normal, non-transformed potato root easily on PGR-free MS medium but not in the presence of Km. Thus for putative transgenic plants rooting medium is supplemented with Km, since only transformed shoots constitutively expressing the *npt*II gene can produce a root system in the presence of Km.

PROTOCOLS – EARLY STUDIES

The early stage studies were conducted in the first half of the 1980s. They comprise studies using wild type strains of *Agrobacterium tumefaciens* and *A. rhizogenes*. The goal of these studies was to elaborate procedures and show that gene transfer in potato is feasible.

The appearance and success of *Agrobacterium*-mediated transformation solved a major problem of potato biotechnology. Initially, a number of transformation techniques were proposed and made available with protoplast transformation as the most promising one. Unfortunately, high somaclonal variation prevented potato protoplasts to become standard recipient target cells for transformation. Thus *Agrobacterium*-mediated transformation was quickly adopted as the main line of transgene research in potato.

According to our literature sources, the first *Agrobacterium*-mediated transgene potato plants were produced by Ooms *et al*. in (1983) at the Rothamsted Experimental Station, UK.

1. Ooms *et al*. (1983)

Shoot cultures of several cultivars including 'Maris Bard', 'Maris Piper', 'Désirée', 'King Edward', 'Pentland Crown' and 'Record' growing on MS PGR-free medium with 2% sucrose were inoculated by wounding with *A. tumefaciens* wild strains T37, LBA 4060 and LBA 1501. Tumors which developed at the site of wounding were excised after three months and subcultured on fresh medium with 200 mg/l Carb to suppress bacterial growth. Cultures regenerated shoots spontaneously in the next two months. Some of the shoots were grafted on stems of untreated, control potato. More detailed studies were done with shoots regenerated from tumors of 'Maris Bard'. Some plants were found to be aneuploid with 47 chromosomes. However, most of regenerated shoots had normal chromosome numbers and they were morphologically identical to the parental line. This finding was in sharp contrast to the results obtained with protoplast-derived plants where high variation was a general rule. Authors concluded that it is most likely that transformed plants almost identical to the parental plants can be obtained from shoots regenerating from *Agrobacterium* induced tumors.

One of the transformants, Mb1501B, was later studied in detail, particularly its endogenous hormonal content and the tuberization ability (Ooms and Lenton 1985). Shoot cultures of Mb1501B cultured *in vitro* contained cytokinins at 100-200 fold higher levels than normal, non-transformed plants. High cytokinin levels somewhat decreased when Mb1501B shoots were grafted onto normal plants. Interestingly IAA levels in the transgene did not differ from levels in normal plants.

2. Ooms *et al*. (1985)

These authors investigated transformation of 'Désirée' using *A. rhizogenes*, strain LBA 9402 with pRi1855. Hairy roots obtained by transformation were induced to form callus from which shoots and whole plants were regenerated. The transformation protocol was the same as in their previous study (Ooms *et al*. 1983). Bacterial inactivation was done with 200 mg/l Carb or Cef. Hairy roots which appeared at the inoculation site were excised and cultured on MS medium with 2% sucrose and 0.12 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) and 2.25 mg/l Zea for 8 weeks to induce callus. Shoots were induced from resulting callus after six weeks on MS medium with 3% sucrose and 0.025 mg/l BA and 10 mg/l GA_3. Shoot cultures upon transformation produced abundant hairy roots. When excised, hairy roots grew vigorously, manifesting intensive branching. Similarly, transformed plants grown in soil in a growth chamber grew more vigorously then the untransformed controls. However, at maturity (after three months), both transformed and controls plants were of equal size. Transformed tubers were longer and contained more eyes than untransformed controls. One of the Ri-transformed (*A. rhizogenes*-transformed) plantlets, D9X8a was later analyzed in detail (Ooms *et al*. 1986) showing organ-specific differential expression of nine T_L-DNA transcripts indicating developmental regulation of gene expression.

In general the Ri-transformed plants manifested surprisingly uniform growth (Ooms *et al*. 1985) although some of them differed in chromosome numbers. Authors concluded that Ri plasmids could be used as vectors for introduction of foreign genes into potato (Ooms *et al*. 1983).

Apart from the early studies on *Agrobacterium*-mediated transformation, Rothamsted Experimental Station hosted important studies on potato protoplast cultures and their stability and somaclonal variation (Thomas 1981; Karp *et al*. 1982; Thomas *et al*. 1982; Fish and Karp 1986).

The early transformation studies of potato were successful showing that the transfer of foreign genes is possible both with *A. tumefaciens* and *A. rhizogenes*. More importantly, apart from some aberrants, fairly uniform regenerants were obtained. It seemed that the bottleneck imposed by somaclonal variation could finally be surmounted.

A study by Horsch *et al*. (1985) made a significant impact on transgenic potato research. The authors showed that in many different plant species leaf explants could be used as excellent starting material for *Agrobacterium*-mediated transformation. With an appropriate PGR combination and balance, leaf explants could directly regenerate transformed shoots avoiding the potentially risky callus stage.

PROTOCOLS – ROUTINE RESEARCH

3. An *et al*. (1986)

This is the first report on transformation of potato using the binary vector system. Binary vector pGA472 and helper plasmids pTiBo542 or pAL4404 were used also to transform tobacco, tomato and *Arabidopsis thaliana*. The explant sources were leaves and shoots of *in vitro*-cultured plants co-cultured for 2 days in 2 ml liquid MS medium containing 10^8 bacterial cells. Explants were then washed off with liquid MS medium and cultured further on callus and shoot induction media containing 200 mg/l Km and 500 mg/l Carb. CIM contained 2.0 mg/l 2,4-D, 0.5 mg/l BA and 15 mg/l glutamine while SIM contained 0.5 mg/l BA and 15 mg/l glutamine. Transformed potato calli expressing the *npt*II gene grew on Km-supplemented medium. From these calli some Km-resistant shoots were regenerated, developing into whole plantlets. Leaf explants were a much better choice for transformation studies than stem explants.

4. Shahin and Simpson (1986)

These authors developed a complex protocol based on the use of leaf disc explants and *A. tumefaciens* strain LBA 4404 containing disarmed vector pARC 8. Prior to bacterial inoculation leaves were pre-cultured for 8 days on medium with 1.2 mg/l NAA + 0.4 mg/l BA resulting in callus proliferating at the time of infection. Co-cultivation lasted for two days on liquid medium with 10^6 bacteria/ml. Co-cultivation medium was replaced with potato culture medium with 250 mg/l Cef for 3-4 days. Disks were then removed, cut in half to increase the exposure to Km and placed on agar-solidified selection medium containing 50 mg/l Km

and 250 mg/l Cef. Selection medium contained 3% sucrose, 40 mg/l AS, 0.18 mg/l NAA (naphthyleneacetic acid), 0.45 mg/l BA, and 50 mg/l CH.

After three weeks green Km-resistant callus was visible attached to disintegrating leaf explants. The transformed nature of this callus was confirmed by an *npt*II activity test. To induce shoot differentiation transformed callus was transferred to medium containing 100 mg/l Cef. After 3 weeks regenerating shoots were placed on medium in which sucrose was replaced with glucose. Shoots were further transferred to media for shoot elongation containing 0.17 mg/l GA_3 instead of IAA and 50 mg/l Cef. Whole plants were obtained and their transgenic nature was confirmed by assaying various organs for *npt*II activity.

This study showed that it is possible to use *Agrobacterium* to transfer genes into potato. However, the procedure was over-elaborated. There are too many steps in the procedure, each one with a different medium and with ample use of callus tissue which could not guarantee genotype stability of transformants. The authors claimed 18 transgenic calli and a number of regenerated transgenic plants.

5. Sheerman and Bevan (1988)

This is a fast, single-step transformation procedure with tubers discs (slices) as explants for transformation. Production of aberrant plants was low (1%) but with a marked genotype effect since two out of 5 investigated cultivars failed to regenerate shoots.

Searching for a combination supporting direct shoot regeneration the authors first investigated responses of different explant types exposed to various PGR combinations and balances. The intention of this approach was to avoid production of callus. Medium with 1.75 mg/l ZR and 0.9 mg/l IAA-aspartic acid (3C5ZR) promoted high direct shoot regeneration in tuber disks of cultivars 'Désirée' and 'Pentland Dell'.

For transformation tuber disks explants (1 cm × 1-2 mm) were floated for 20 min in 20 ml MS medium with bacterial suspension and then placed on media with tobacco FCLs over 3C5ZR medium for 2 days. Explants were then transferred to 3C5ZR medium with 100 mg/l Km and 500 mg/l Carb. Plates sealed with parafilm and cultured in light at 25°C were subcultured at 3-week intervals. Carb was decreased to 200 mg/l. Shoots started to regenerate 4-6 weeks after the inoculation. For rooting shoots were transferred to PGR-free medium with 200 mg/l Carb and 100 mg/l Km.

Shoot regeneration efficiency was 6-20% for tuber explants of 'Désirée' 'Pentland Dell' and 'Golden Wonder'. 'Maris Bard' and 'Maris Piper' failed to regenerate shoots. Rooting was 80% efficient; roots appeared from nodes and not from the cut surface. Untransformed shoots did not form roots. Among 200 analyzed transgenic plants, only 1% were found to differ from the original, parental stock. Authors concluded that direct (single step) regeneration of shoots decreases the possibility of aberrant plant formation.

6. Stiekema *et al*. (1988)

This excellent study appeared concomitantly with the study of Sheerman and Bevan (1988). Both protocols used tuber disk explants, with cell suspensions as feeder cell layers during inoculation and Zea-based cytokinins for callus/shoot induction. The Stiekema *et al*. (1988) protocol is a two-stage protocol in contrast to the single-stage protocol of Sheerman and Bevan (1988).

Stiekema *et al*. (1988) optimized their protocol for transformation of leading cultivars, 'Bintje' and 'Désirée' using tuber explants as starting material. *A. tumefaciens* strain LBA4404 was used with helper plasmid pRAL4404 and pBI121 containing a *gus* gene with CaMV 35S promoter. Explants excised with a 10 mm cork borer were cut into 3 mm thick slices.

Discs were placed on 'Bintje' FCLs on medium with 1.0 mg/l Zea and 0.01 mg/l NAA for three days at 24°C and 16 h light. Discs were moved to medium with the same PGR composition without FCLs but with the addition of 50-100 mg of Km and 200 mg/l Cef for two weeks. For shoot induction explants were transferred to MS medium with 0.25 mg/l BA and 0.1 mg/l GA_3 and Km for 2-3 weeks. For rooting, MS medium with 2% sucrose was supplemented with 0.1 mg/l IAA and 50-100 mg/l Km. Transgenic plants were screened for β-glucuronidase activity.

This protocol is fast, enabling early rooting of transgenic shoots some 4-6 weeks after the start of inoculation. However, this is slightly over-exaggerated since shoots used for rooting are actually 1-2 mm long shoot primordia. Each tuber disc typically regenerated 2-5 shoots. Leaf discs and stem explants were also investigated but their regeneration capacity was much lower in comparison to tuber discs. Authors estimated that following the co-cultivation and selection with Km only about 1% of tuber disc slices gave rise to Km-resistant shoots. Three transgenic 'Bintje' plants (BIGUS I–III) and four out of six transgenic 'Désirée', plants (DEGUS I–VI) appeared normal. DEGUS III and VI showed morphological differences in leaf morphology – leaves were narrow and small. DEGUS III was aneuploid with 47 chromosomes while other plants were euploid with 48 chromosomes.

7. de Block *et al*. (1988)

This protocol using leaf disc explants was modified from the protocol of Horsch *et al*. (1985). It is a fast protocol producing rooted transgenic plants in some 7 to 10 weeks with almost no somaclonal variation. Cultivars investigated were 'Bintje', 'Berolina', 'Désirée' and 'Russet Burbank', previously established as shoot cultures. Transformation was done with C58C1 and various plasmids and promoters. Marker genes were *npt*II and *bar*.

Explants were 3-10 mm long leaves from 3-4 week old plants. For inoculation they were floated upside down in infection medium with bacteria for 2 days. This was a MS PGR-free medium with 3% sucrose, 2% mannitol, buffered with 0.5 g/l MES and with 30 µl of bacterial suspension per plate. The addition of PGRs to the infection medium was not advantageous. Leaves of 'Russet Burbank' perished in this treatment.

Explants were washed in infection medium with 1000 mg/l Carb or 500 mg/l Cef. They were blotted dry and further cultured on a complex CIM medium containing 1.0 mg/l *trans*-Zea, 0.1 mg/l NAA, 40 mg/l AS, 200 mg/l glutamine, 2% mannitol, 2% glucose instead of sucrose and 1000 mg/l Carb or 500 mg/l Cef and 50-100 mg/l Km. Good ventilation of plates with explants was required. Explants were subcultured to fresh medium after a week. After two more weeks many small calli formed on wound edges of leaf explants and they were transferred to SIM 1 medium, same as CIM but with NAA removed and halved concentration of antibiotics. After 2-3 more weeks leaf explants with calli were transferred to SIM2 medium same as SIM1 but containing also 0.01 mg/l GA_3 and 250 mg/l Carb or 150 mg/l Cef. Regeneration of 'Russet Burbank' was possible only with supplementation of filter sterilized 10.0 mg/l $AgNO_3$.

Trans-Zea could be replaced with BA but it produced less shoots. After a month nearly every callus regenerated shoots. Selection with Km was far better than with phosphinotricin. Transformation frequency with Km was 100% compared to 20% with the use of phosphinotricin. All investigated transgenic plants were resistant to herbicide Basta. Southern analysis was done with 9 putative transgene plants; three of them contained 2 T-DNA copies while the remaining 6 plants contained a single copy. Chromosome count of 40 plants (10 plants of each variety) showed that only one 'Berolina' plant had 47 instead of expected 48 chromosomes.

de Block's (1988) protocol can be considered a three-step protocol since there are three separate callus + shoot regeneration media designated as CIM, SIM1 and SIM2. Although the original protocol is quite complex, many sim-

plified modifications are still in use. We had very good experience with such modifications success transforming various cultivars.

8. Tavazza *et al.* (1988)

This protocol was developed specially for 'Désirée'. Explants were 6 mm diameter leaf discs prepared from 3-4 weeks old potato shoot cultures. Leaf explants were precultured on feeder cell layers a day before inoculation in an inverted position. Feeder layers consisted of 9-days old 'Désirée' cell suspension cultured on UM medium with 0.25 mg/l kinetin and 5.0 mg/l 2,4-D. Medium containing nurse cells was covered with Whatman #3 MM paper two days before culturing leaf discs. Inoculation of explants in an overnight culture of bacteria was done by short dipping lasting 1-2 min with gentle shaking. Leaf explants were blotted dry and returned to the feeder plates. After 2 days explants were transferred to shoot induction medium containing MS with 5% sucrose, 1.0 mg/l BA, 1.0 mg/l IAA, 10 mg/l GA_3 and 200 mg/l Cef and Vanc to eliminate *Agrobacteria* and 100 mg/l Km for selection of transformed tissue. Regenerated shoots were transferred to PGR-free medium with 1% sucrose.

The authors observed that the incubation time inversely affected the transformation frequency (efficiency). Thus, inoculation lasting only 1-2 min was better than 5-10 min. The feeder cell layer culture affected the transformation process by shortening the time required for regeneration of transformed shoots. Using feeder layer techniques and LBA 4404/pGA492 for inoculation, callus started to develop after 2 weeks and shoots appeared within 3-4 weeks. Shoots appeared directly from Km-selected tissue or calli at a frequency of 23%. Each leaf disc usually produced 4-5 shoots. In the same conditions explants inoculated with A136 became necrotic and died. In the absence of Km these explants also manifested high shoot regeneration potential. Transformation success decreased at temperatures higher than 19°C and with explants collected from plants older than 3 weeks. Transformed plants were morphologically identical to normal 'Désirée' plants. Transformation was confirmed by Southern blotting for the presence of the *npt*II gene.

The main characteristic of this protocol was rapid regeneration of transformed shoots accompanied with minimal callus formation and uniformity of regenerated plants. A disadvantage is the use of feeder cell layers which complicates the protocol.

9. Wenzler *et al.* (1989)

Potato line FL1607 was transformed with *A. tumefaciens* LBA 4404. Plasmids contained CaMV-GUS construct (pBI121) or class-I patatin clone PS20 fused to a *gus* reporter gene (pPS20A-G). Explants were leaf strips 2-3 mm wide excised from 3-4 week old shoot cultures. Explants with their abaxial sides were placed on stage 1 medium containing 2.24 mg/l BA + 0.2 mg/l NAA + 10 mg/l GA_3 for four days. After precultivation, explants were inoculated for 10 min in diluted 10^7 bacteria/ml bacterial solution, blotted dry and returned to the precultivation medium. After 3-4 days explants were transferred to the same medium supplemented with 500 mg/l Carb and 50 mg/l Km on which they stayed 12 days. Shoots were induced on stage 2 medium containing exactly the same as stage 1 medium except for auxin and the addition of antibiotics.

Two weeks after inoculation FL1607 leaf explants produced compact yellow-green callus at cut edges. Following the transfer to stage 2 medium shoots were regenerated after 4 weeks. Three cultivars were tested: 'Désirée', 'Superior' and 'Russet Burbank'. Their explants were far less responsive to this procedure. Thus while FL1607 produced 400-500 shoots per 100 explants, 'Désirée' produced only 20. 'Superior' and 'Russet Burbank' failed to produce shoots with this procedure.

At 50 mg/l Km 65% of regenerated shoots transformed with CaMV-GUS were GUS-positive. With the patatin-GUS construct 60% of regenerants expressed GUS activity. Authors concluded that the production of transformed potato plants is primarily dependent on the genotype.

10. Hänish ten Cate *et al.* (1988)

This is an *A. rhizogenes*-mediated transformation protocol of 'Bintje' and 'Désirée' with strains LBA9402 and AR15834. Leaf segment and tuber disc explants were inoculated after Annand and Heberlein (1977). On tuber disc explants roots developed randomly on the surface without visible callus formation. On leaf explants roots developed only above leaf veins. Hairy root clones had variable phenotypes. Differences between the two bacterial strains were not observed. Shoot regeneration was achieved according to Ooms *et al.* (1985) and was achieved in about 10% of hairy root lines. Shoots could regenerate directly from roots but in most cases they appeared from compact green callus within 3 weeks. All lines were tetraploid, there were no aneuploid plants.

All *Ri*-transformed 'Désirée' and most of the 'Bintje' plants were bigger and grew more vigorously than normal type plants. Most plants were as expected tetraploid. *Ri*-Bintje plants transplanted into soil showed variation not visible during *in vitro* growth. There were variations in plant height, tuber yield, apical dominance, leaf form and many other traits. *Ri*-Désirée plants were more uniform, tall with strong apical dominance and normal leaves. Variation was observed in tuber yield and form, mainly among root lines.

11. de Vries-Uijtewaal *et al.* (1988, 1989)

Transformation was done with *A. rhizogenes*, strains LBA9402 (pRi1855); LBA9365 (pRi8196); and *A. tumefaciens* LBA 1020 (= LBA285 + pRi1855::Tn5). Plant material: 8 haploid and 2 diploid potato lines.

The explants were 5 mm-long stem internodes since under normal conditions they never produce roots. Inoculation was in 1/10 overnight bacterial culture in liquid YMB for 60 min. Explants were washed, blotted dry and placed on PGR-free medium for 24 h then washed again in MS + 5% sucrose + 200 mg/l Cef and placed on the same agar-solidified medium for 3-4 weeks. Roots were isolated and cultured as separate clones on the same medium.

The first hairy roots were visible after 10 days and their number increased with time. There was no intermediate callus formation. Genotypes differed in response to hairy root formation. Four different LBA9402 phenotypes were recognized, a characteristic that was constant for the genotype. Efficiency of hairy root production was highly variable, in diploids it was over 80% and in monohaploids it was usually less then 40%. Genetic stability was expressed as the maintenance of the original ploidy level and was high. Shoot cultures of monoploid lines also contained diploid and tetraploid cells and it seems that only these higher ploidy cells were transformed.

12. Ottaviani *et al.* (1990)

This was an optimization of the *A. rhizogenes* transformation protocol for 'Bintje', finding answers for questions which needed empirical confirmation. In all explants it was possible to obtain roots directly without callus formation. Root transformation was done according to Annand and Heberlein (1977) and shoot regeneration according to a modified procedure of Ooms *et al.* (1985). Callus induction was done on medium containing MS + 2% sucrose + 1.0 BA + 0.2 2,4-D for three weeks, and shoot induction on MS + 3% sucrose + 1.0 mg/l Zea + 1.0 mg/l GA_3 for three weeks. Shoots were later multiplied on PGR-free media with 2 and 3% sucrose.

Hairy roots originated from cambial cells of vascular bundles. Among different explant types leaf and stem explants were superior in shoot regeneration to tuber explants. Shoots appeared in 60% of root clones induced on stem and

leaf explants compared to 25% on clones induced from tuber disc explants.

Authors performed optimization of shoot regeneration investigating separately the effects of auxins, cytokinins and sucrose. They claim that sucrose concentration needs to be reduced to 0.5-0.1% in order to maintain healthy green color of callus. Incidentally, at such low sucrose concentration callus did not regenerate shoots. Similarly, NAA is an auxin suitable for callus induction but later it did not support shoot regeneration. Among auxins 2,4-D was the best choice for callus induction.

Medium recommended for callus induction contained MS + 1.5% sucrose + 3.0 mg/l Zea/ZR + 0.05-0.1 mg/l 2,4-D. For shoot regeneration medium contained MS + 3% sucrose + 3 mg/l ZR + 1.0 mg/l GA_3.

13. Visser *et al*. (1989a)

These authors studied transformation using a number of different *Agrobacterium* strains containing *A*. *rhizogenes* plasmids. Inoculation was done by a 15-min immersion of stem segments in bacterial suspension and their placement on PGR-free MS medium + 3% sucrose after washing and blotting dry with Whatman #3 MM filter paper. After 2 days explants were transferred to same medium supplemented with 200 mg/l Cef. Roots appeared after 10 days. They were excised and cultured on PGR-free MS medium with 3% sucrose, 200.0 mg/l Cef and 100 mg/l Km for several weeks.

For callus induction pieces of hairy roots were cultured on MS medium with 2% sucrose + 2.0 mg/l Zea+ 0.12 mg/l 2,4-D + 200 mg/l Cef and 50 mg/l Km until green callus clumps reached an area of 15 mm^2. Medium for shoot induction contained MS + 3% sucrose + 2.25 mg/l BA + 10 mg/l GA_3 + 200 mg/l Cef and 50 mg/l Km for 2/3 weeks. Shoots were further cultured on PGR-free MS medium with 3% sucrose and 200 mg/l Cef.

Authors considered that the transformation procedure supported good ploidy level stability of diploid and tetraploid plants. They concluded that *A*. *rhizogenes* transformation was superior to *A*. *tumefaciens* transformation.

14. Visser *et al*. (1989b)

This is a report on *A*. *tumefaciens*-mediated transformation of amylose-free mutant 86.040 and dihaploid 79.7932. Bacterial strain was LBA4404 with pVU1011. Vector allowed selection for Km and Hygro-resistant transgenic plants.

Stem and leaf explants were precultured overnight in MS medium with 10.0 mg/l BA + 10 mg/l NAA + 80 mg/l ammonium nitrate and 14.7 mg/l calcium chloride. For inoculation, explants were immersed 15 min in an overnight bacterial suspension. They were blotted dry with sterile filter paper and placed on MS medium with 0.1% sucrose + 0.4% mannitol + 2.25 mg/l BA + 0.0175 mg/l IAA for 2 days. Explants were then transferred to the same media after the addition of 200 mg/l Cef with or without 50 mg/l Km. Explants were further transferred to shoot induction medium, MS + 1.5% sucrose + 2.25 mg/l BA + 5 mg/l GA_3 with 200 mg/l Cef and 50 mg/l Km. Subculturing was done at 4-week intervals. After 4 weeks 70% of leaf explants regenerated shoots and this percentage increased to 85% after 6 weeks. In shoot explants regeneration, although initially slower, reached the same values as in leaf explants after 6 weeks. Addition of Cef did not affect the regeneration of shoot and leaf explants. However, Km was detrimental and it was better to start Km selection later, after some 5 to 7 days. Untreated leaf and stem explants could not multiply shoots in the presence of 10 mg/l Km.

Forty shoots were micropropagated and planted into soil. The morphology of 35 plants was identical to untransformed control plants of both investigated lines. Five plants were abnormal; four differed only in leaf shape while the fifth was highly malformed.

Transformation efficiency measured as the percentage of Km resistant plants was 6-8% with delayed selection. Shoot explants were more efficient in producing Km-resistant shoots. Among the seven investigated plants DNA analysis showed more than 1 copy T DNA insertion in 4 plants.

PROTOCOLS – INTRODUCTION OF GENES OF INTEREST

15. Rocha-Sosa *et al*. (1989)

This is a simple and fast transformation protocol written briefly providing almost no details on explant inoculation and shoot regeneration. Authors studied expression of a patatin-class I gene which was isolated and incorporated into the binary pBin 19 vector of *A*. *tumefaciens* strain LBA 4404. The construct contained the *gus* gene from *E*. *coli* and *npt*II reporter gene. Expression of this construct was induced by increased sucrose content of the media requiring sucrose to be replaced by glucose used at low concentration (1.6%). A shift to high sucrose nutrition was accompanied with a 20-100-fold increase of GUS activity.

This simple, single-step protocol became very popular in studies of carbohydrate metabolism of transgenic potato plants. Unfortunately it has some mistakes in media component concentrations (GA_3). Luckily, a similar study by Keil *et al*. (1989) seems to utilize the same, properly presented protocol.

Protocol was optimized for 'Désirée' and 'Berolina', using leaf discs, inoculated 2 days in darkness. Regeneration medium contained 2.0 mg/l ZR, 0.02 mg/l NAA, 20.0 mg/l GA_3, 500 mg/l Claf, 50 mg/l Km and 1.6% glucose instead of sucrose (from Keil *et al*. 1989). Explants were subcultured every 10 days. After 3-4 subcultures shoots were excised and transferred to PGR-free MS medium with 2% sucrose and 250 mg/l Claf. At least 40 shoots/plants were produced in independent transformation events.

The Rocha-Sosa *et al*. (1989) protocol created a precedence used in many later studies mostly on the carbohydrate metabolism of transgene potato plants. It actually enabled later studies to be published providing minimal information on the transformation procedure.

16. Newell *et al*. (1991)

This paper, prepared by a group of Monsanto scientists, presents research on 'Russet Burbank' equipped with resistance against potato viruses. As a complement to this study we recommend an article by Kaniewsky and Thomas (2004) who described problems encountered during the production of first commercial transgene potato cultivars at Monsanto.

This is a complex 2-stage procedure using also FC layering through the inoculation stage. Explants were collected from shoot cultures maintained on a medium containing MS + 3% sucrose + 0.4 mg/l thiamine HCl + 170 mg/l $Na_2HPO_4 \cdot H_2O$ solidified with 0.2% Gelrite.

Explants for transformation were 5-10 mm long internode segments with axillary buds removed. Cut ends were smeared with 3-day old bacterial plate cultures. Inoculation by co-cultivation was done for 2 days on medium containing 1/10 strength modified, PGR-free MS (Jarret *et al*. 1980a, 1980b) with 0.1% sucrose and tobacco cell feeder layer. Explants were transferred to (CIM) containing 3.0 mg/l BA + 0.01 mg/l NAA + 100 mg/l Km + 500 mg/l Carb for 4 weeks and then to P2 medium (SIM) containing 0.3 mg/ GA_3 + 100 mg/l Km + 500 mg/l Carb for 4 more weeks. Rooting was done on PGR-free medium with 500 mg/l Carb. Subculturing was done at monthly intervals. This same procedure was successfully used on 'Désirée' and 'Kennebec' providing better transformation efficiency than with 'Russet Burbank'. Authors considered that shoot explants are superior explant type in comparison to leaves (de Block 1988) and microtubers (Ishida *et al*. 1989) since they produce more shoots in less time and do not require the presence of silver nitrate. Attempts to produce transgenic plants from 'Russet Burbank' tuber discs by the procedure of Sheerman and Bevan (1988) were unsuccessful.

17. Edwards *et al.* (1991)

This study is dedicated to 'Désirée' transformed to express pea lectins. Explants consisting of 7 × 7 mm leaf squares were incubated in bacterial suspension for 30 min with gentle agitation. They were blotted dry and placed abaxial side down in contact with the medium with 2% sucrose, 0.5 mg/l Zea and 2.0 mg/l 2,4-D for 2 days. Explants were washed overnight in the same medium + 100 mg/l Augm and then placed on the same medium with 100 mg/l Km for 4 more days. Explants were then moved to shoot induction media containing MS + 2% sucrose + 0.5 mg/l Zea + 2.0 mg/l GA_3 + 100 mg/l Augm and 100 mg/l Km. After 6 weeks regenerated shoots were transferred to shoot maintenance, PGR-free MS medium with 2% sucrose.

This procedure enables efficient and rapid production of phenotypically normal potato plants from leaf mesophyll tissues and was later adopted mostly by authors working on potato metabolism.

18. Dietze *et al.* (1995)

This is a two-stage transformation protocol elaborated for 'Désirée'. Bacterial type, strain and plasmid data were not provided. Explants are leaves placed upside down on top of MS medium with 2% sucrose and 50 µl of bacterial culture spread over the surface suspension for 2 days in darkness. Explants were then transferred to callus induction medium MS + 1.6% glucose + 0.1 BA + 5 mg/l NAA + 250 mg/l Claf + 50 mg/l Km or 1 mg/l Hygro for 7 days. Then transfer explants to shoot induction medium containing 2.0 mg/l ZR + 0.02 mg/l NAA + 0.02 mg/l GA_3 + 250 mg/l Claf + 50 mg/l Km or 1 mg/l Hygro. Shoots appeared in the second subculture. Then they were transferred to root induction medium, i.e. plain MS medium with Claf. It seems that all shoots which regenerated under selection pressure were transformed. The SIM medium used in this protocol is same as the CIM medium of Rocha Sosa *et al*. (1989). Two to three transformants per leaf were obtained. Km selection took 7-8 weeks to produce the first 50% of totally produced shoots. Hygro selection took more time with about 3-4 transformants per leaf. The protocol is same as the previous one (Rocha-Sosa *et al*. 1989) popular in studies of metabolism of transgene plants.

19. Kumar (1995)

This protocol, used for transformation of five wild *Solanum* species based on microtuber explants, is a modification of Sheerman and Bevan's (1988) protocol. Microtubers were sliced into 1-mm thick discs. They were briefly (30 min) precultured in a simple liquid MS medium prior to the addition of bacterial suspension. Co-cultivation lasted 30 min; explants were blotted dry and cultured on MS + 2% sucrose + 1.8 mg/l ZR + 0.9 mg/l IAA aspartic acid for 2 days. Explants were transferred to the same media supplemented with 150 mg/l Km and 250 mg/l Cef, and subcultured every 2 weeks.

Shoot regeneration was fast and efficient, providing 3-4 shoots per explant disc within 2-4 weeks, although a simple liquid medium preculture was performed instead of FC layering (Sheerman and Bevan 1988). However, shoots that regenerated first were non-transformed escapes developing from preexisting eyes, and true transformants appeared only after 3-4 weeks. Transgenic plants were morphologically normal and indistinguishable from the control parental plants. Transformation frequencies ranged from 2.0% in *S. stoloniferum* to 9.6% in *S. papita*. It was 3.2% in *S. verrucosum*, 4.2% in *S. hjertingii* and 5.8% in *S. demissum*.

20. Dobigny *et al.* (1995)

Transformation of two French cultivars, 'Fanette' and 'BF15' was achieved with cucumopine, mannopine and agropine strains of *A. rhizogenes* (15834, 2659, 2659GUS and 8196GUS) with plasmids pRi15834, pRi2659, pRi 2659/pBI121-1 and pRi 8196/pBI 121-1. Stem internode explants 5-mm long were smeared with bacteria harvested with a spatula from agar plates, spread on top of stem fragments and placed on MS medium with 500 mg/l Cef. Roots emerging from the infection site were excised (one root per explant) and cultured on basal MS medium. Root subculturing was done every four weeks.

Root formation in both potato cultivars was poor indicating that mannopine and cucumopine strains are unable to induce hairy root formation in potato. Thus, when functional genes coding for auxins are not present in the transferred T-DNA, rooting is possible only with the addition of auxins. Root formation was efficiently increased after applying a pretreatment of up to 5.0 mg/l NAA prior to bacterial inoculation. Using NAA pretreatment stable transformed roots were obtained with a frequency reaching 85%.

Roots cut into 5-mm long segments were placed for 3 weeks on MS medium with 0.05 mg/l 2,4-D to produce callus. For shoot regeneration they were transferred to SIM containing 2.0 mg/l Zea, 1.0 mg/l BA and 3.24 mg/l GA_3. Whole plants were regenerated from 80% selected root lines within 6 weeks on the regeneration medium. Transformation of selected plants was confirmed by GUS activity and the presence of opines.

21. Beaujean *et al.* (1998)

This protocol contains improved procedures for transformation of 'Désirée', 'Bintje' and 'Kaptah Vandel', with a PGR combination and balance decreasing the somaclonal variation and appearance of aberrant plants. Transformation was done with C58C1Rif1 containing pGS gluc 1 which harbors *npt*II and the *gus* reporter gene.

Internode segments 6 mm long were longitudinally cut split in half and treated for 30 min in 1/10 bacterial suspension. After blotting dry explants were positioned for three days on CIM containing 1.0 mg/l BA, 0.1 mg/l NAA and 0.1 mg/l GA_3. Explants were washed for 30 min in liquid MS containing 1000 mg/l Cef and then positioned on callus inducing medium supplemented with 250 mg/l Cef and 125 mg/l Km for 4 weeks. Medium for shoot induction contained 1.0 mg/l BA, 0.1 mg/l GA_3, 125 mg/l Km and 300 mg/l Cef for 4 weeks. For rooting green shoots 1-2 cm long were transferred to medium containing 0.1 mg/l IAA, 80 mg/l Km and 200 mg/l Cef. Visual inspection revealed morphological abnormalities in 8 out of 150 acclimatized plants. Flow cytometry revealed 17-19% of plants showing abnormal ploidy levels. Activity check included GUS assay, *npt*II-specific PCR and Northern hybridization.

Since there was significant aberrant plant formation the study was continued in a search for a protocol with less somaclonal variation. Several different cytokinins were tested until it was found that replacing BA with ZR stops aberrant plant production. A corrected procedure used the following media. For callus induction 0.8 mg/l ZR + 2.0 mg/l 2,4-D for 9 days; for shoot induction 0.8 mg/l ZR + 2.0 mg/l GA_3 + 125.0 mg/l Km + 300 mg/l Cef; for root induction 0.1 mg/l IAA + 80 mg/ Km + 200 mg/l Cef. Transformation efficiency was 88.7% for 'Désirée', 95.2% for 'Bintje' and 74.7% for 'Kaptah Vandel'. The protocol supported high bud production, 7-9 buds per explant in a short time (4-6 weeks).

Flow cytometry did not detect plants with abnormal ploidy levels. Visual inspection also failed to detect abnormal plants.

22. Trujillo *et al.* (2001)

This is a single step transformation protocol optimized for Andean potato cultivars 'Diacol Capiro' and 'Parda Pastusa' popular in Colombia. Transformation was done with LBA 4404 and pBI-121. Leaf explants were precultured for 1-3 days (Visser 1991) and then they were immersed for 10 min in 1:50 (10^8 bacterial cells/ml) bacterial suspension, blotted

dry on sterile filter paper and placed with abaxial surface in contact with the single step shoot regeneration MS medium with 3% sucrose, 0.05% CH, 3.0 mg/l ZR, 1.0 mg/l GA_3 and 40 mg/l ascorbic acid. After 3 days explants were transferred to the same medium supplemented with 100-150 mg/l Km and 500 mg/l Carb and kept in darkness for 15 days. Explants were then exposed to light until they produced calli and regenerated small shoots. Shoots reaching 1 cm in length were excised and cultured on PGR-free MS medium with 3% sucrose, 2 mg/l D-pantothenate and 3 mg/l sodium thiosulfate.

23. Barell *et al.* (2002)

This protocol is focused on the use of different selective agents. The authors created several new binary vectors designed pMOA1 to pMOA5 each differing only in the selective agent. Vectors conferred resistance to Km (*npt*II), Hygro (*hpt*), methotrexate (*dhfr*), phosphinotricin (*bar*) and phleomycin (*ble*).

The T-DNA size of all the vectors used in this study was small. It was based on the minimal features necessary for plant transformation, with no extraneous DNA, containing only a series of unique restriction sites between the right border and each selectable marker gene for subsequent insertion of useful genes. Selective agents used in this study are presented in (**Table 3**).

Plants used for explants were grown on potato multiplication medium containing MS salts, 3% sucrose, 40 mg/l ascorbic acid and 500 mg/l CH. Leaves cut in half were dipped for 30 sec in liquid *Agrobacterium* culture and then blotted dry on sterile filter paper. Explants were transferred to CIM containing 2.0 mg/l BA and 0.2 mg/l NAA. Two days later 200.0 mg/l Tim was added and then 5 days later one of the five selection agents. After some 2-6 weeks small cell colonies were transferred to shoot regeneration medium containing sucrose reduced to 0.5%, 1.0 mg/l Zea, 5.0 mg/l GA_3, 200 mg/l Tim and the selective agent. Shoots were excised and rooted on PGR-free medium with 100 mg/l Tim. Callus and shoot induction were performed at subdued light (5-10 μmol m^{-2} s^{-1}). Standard irradiance for maintenance of shoot cultures was 80-100 μmol m^{-2} s^{-1}.

Efficient transformation required selection to be delayed for 5 days, enabling cells at the cut end of leaves to form small callus proliferations. Km was the best selective agent, followed by Hygro while metotrexat resistance was the least preferred option; phosphinotricin and phleomycin resistance were intermediary. Phosphinotricin resistance provides false-positive shoots unless CH is removed from the medium. This was presumably a consequence of sufficient glutamine present in CH. Authors also consider that replacement of BA used in their previous study (Conner *et al*. 1991) with Zea was a significant improvement of the transformation procedure. They also consider that dying, necrotic tissue may have a negative effect on transformed cells requiring early removal of regenerated callus from explants cultured on CIM medium.

Table 3 Selection agents and their concentrations used in the protocol Barell *et. al.* 2002 (23).

Selection agent (mg/l)	Callus initiation	Shoot regeneration	Root initiation
kanamycin	100	100	50
hygromycin	25	25	12.5
metotrexate	0.1	0	0
phosphinotricin	10	10	10
phleomycin	0.4	0.4	15

24. Ducreux *et al.* (2005)

This is a protocol for *Solanum phureja* transformation, with LBA4404 and the *crtB* phytoene synthase gene coding for phytoene synthase, which converts geranylgeranyl pyrophosphate to a carotenoid phytoene. Carotenoids have been known as effective in preventing a variety of diseases including cancer and cardiovascular disorders. Mammalian cells cannot synthesize carotenoids and rely on a dietary source for their intake. Explants were precultured in liquid MS + 2% sucrose medium and then inoculated by adding 2 ml of bacterial suspension to each Petri dish for an inoculation lasting 5-10 min. Explants were gently blotted and placed on CIM with 2.5 mg/l ZR + 0.2 mg/l NAA + 0.02 mg/l GA_3 for 2 days and then replanted to same medium + Cef for 12 days followed by a transfer to a SIM medium with 2.5 mg/l ZR + 0.02 mg/l NAA + 0.02 mg/l GA_3 + 500 mg/l Cef and 50 mg/l Km for at least 3 subcultures lasting for two weeks. Shoots were excised and rooted for two sub-cultures on MS + 2% sucrose medium with Cef and Km. Southern analysis showed that transgene copy number varied from one to five. There was a high incidence of chromosome doubling occurring in over 80% of all lines.

25. Heeres *et al.* (2006)

Transformation was studied in 16 potato cultivars using LBA 4404 containing plasmid pKGBA50 with the antisense gene coding for granule-bound starch synthase (GBSS). In transgene clones the amylose production in the tuber was suppressed by the antisense RNA-mediated inhibition of the GBSS gene. Two different transformation protocols were used to establish the best transformation procedure for each variety. Each cultivar was transformed according to both protocols, and after a pilot experiment, large-scale transformations were carried out following the best protocol for any particular variety. Protocol I was essentially done according to the one described by Visser (1991). Protocol II was followed according to Edwards *et al.* (1991). Six cultivars showed better transformation results with protocol I, while the remaining ten cultivars gave better results with the protocol II.

Stem internodes 5-10 mm long were precultured for 1 day in liquid MS medium (Visser *et al*. 1991) and then soaked overnight in a bacterial suspension and co-cultured for 2 days. Explants were then washed with MS with 200 mg/l Km and transferred to CIM. In protocol I CIM contained 1.0 mg/l ZR + 200 mg/l Cef + 100 mg/l Km. In protocol II CIM contained 0.5 mg/l Zea, 2.0 mg/l 2,4-D, 200.0 mg/l Cef and 100 mg/l Km. After 5 days explants were transferred to protocol II SIM containing 0.5 mg/l Zea+ 2.0 mg/l GA_3 + 200 mg/l Cef and 100 mg/l Km. Shoots were considered transgenic after they rooted on MS medium containing 3% sucrose, 200 mg/l Cef and 100 mg/l Km.

There was a large variation in the percentage of regeneration of transgenic shoots, from 0.3% ('Nika') to 55.7% ('Kardal'), and in rooting ranging from 0.2% ('Nika') to 35.0% ('Kardal').

Only 23 of 171 clones did not contain the vector sequences. Vector backbone DNA sequences were assessed by PCR specific to the *npt*III and *trf*A genes. *npt*III is a bacterial Km resistance marker gene for bacterial selection, and *trf*A for mobilization of the plasmids from *E. coli* to *Agrobacterium* via triparental mating.

Some plants showed a weak root development and absence of tubers. Total percentage of aberrant clones in the greenhouse was 10.3% and in the field it increased to 19.6%. The type of deviations seemed to be related to the parent cultivar.

For a number of transformants the amylose-free phenotype, yield and specific gravity were followed during the next 3 or 4 years. From the 903 non-deviating clones in the greenhouse, 90% turned out to be phenotypically comparable to the parental cultivar in the field, whereas 10% showed deviations which were not observed earlier in the greenhouse. Two clones which showed a color/shape deviation in the greenhouse were normal when grown in the field. In general a variety which showed a short period of first shoot appearance, also showed a large number of shoots. The three best regenerating varieties 'Kardal', 'Astarte' and 'Karnico' are related, suggesting a common genetic background in-

heritance for high regeneration capacity. Regeneration and transformation efficiency are two different genetically controlled factors.

26. Gustafson *et al.* (2006)

A group of Canadian authors optimized the transformation protocol for 'Shepody' using LBA4404 with pSOL6 (binary). The starting point in this extensive study was the protocol developed by de Block (1988). The authors first investigated the callus and shoot regeneration capacity of shoot and leaf explants in relation to the NAA/IAA + *trans*-Zea PGR combinations (0, 0.1, 1.0 mg/l of NAA or 0, 0.1, 1.0 mg/l of IAA in combination with 0, 0.1, 1.0, 5.0 mg/l *trans*-Zea). Shoot explants consisted of 5 mm-long internode segments. The leaf tip and basal portions including the petiole were discarded from thick, healthy leaves from upper shoot internodes. Remaining leaf lamina was cut into 5 × 5 mm square explants.

For transformation studies intact leaves with petioles were used. A loopfull of bacteria was placed in infection medium (IM) until an OD_{600} = 0.6 was reached. Explants were immersed in the bacterial suspension with 72.5 mg/l AS for 2 min and then placed on CIM with 1.0 mg/l NAA and 1.0 mg/l *trans*-Zea in low light at 22 ± 2°C for co-cultivation. After 2 days, the explants were transferred to callus selection medium CSM (CIM + 100 mg/l Km + 300 mg/l Cef). CIM was based on the S3 medium of de Block (1988). SIM (called here SGM – shoot generation medium) was the same as CSM (CIM) but with 1.0 mg/l *trans*-Zea and no auxins. All plant growth media contained MS + 3% sucrose.

For callus induction 14 different PGR combinations were investigated separately for shoot and leaf explants. Only one combination (0.1 mg/l *trans*-Zea + 0.1 mg/l NAA) supported shoot regeneration in both explant types. Highest shoot regeneration for leaf explants was on 1.0 mg/l *trans*-Zea + 1.0 mg/l NAA and for shoot explants on 0.15 mg/l *trans*-Zea + 0.1 mg/l IAA.

Among the 9 PGR combination regenerating shoots from callus, the efficiency of the original de Block (1988) procedure was ranked fifth. Applying the optimized transformation protocol on 467 explants 59.5% regenerated shoots/plant in 10-23 weeks. Among regenerated plants 50 (18%) were off-type. PCR confirmed transformation as *npt*II-positive in 47.1% of explants regenerating shoots, bringing the confirmed transformation rate to 28.0%. A total of 450 independent transgene lines were created within a year. With this study 'Shepody' joined the group of cultivars with well elaborated transformation protocols.

27. Banerjee *et al.* (2006)

This is a fast and simple method enabling transformed shoots of *S. tuberosum* ssp. *andigena* line 7540 to appear 4 weeks after the inoculation. Still, some extra time is needed for these small shoots to elongate and some more for plantlets to root. Transformation was done with *A. tumefaciens* strain GV2260 with pCB201 containing a full length cDNA of StBEL5.

Leaf explants from 4 weeks old *in vitro* plants were wounded over the midrib, placed with adaxial side down in Petri dish with 20 ml liquid PGR-free MS medium with 2% sucrose and 100 µl of ice-cold *Agrobacterium* culture. Plates with some 20 leaf explants, gently shaken for 15 min at 35 rpm were sealed and placed in the dark for co-cultivation. There was no washing and blotting before transfer to CIM containing MS + 1.6% glucose, 5 mg/l NAA, 0.1 mg/l BA, 250 mg/l Cef and 50 mg/l Km adaxial side down. After 7-8 days explants were transferred to SIM containing MS + 1.6% glucose, 2.2 mg/l ZR, 0.02 mg/l NAA, 0.1 mg/l GA_3, 250 mg/l Cef and 50 mg/l Km. Shoots were visible 25-28 days after inoculation. Explants were subcultured every 10 days until shoots reached 2-3 cm in length. Shoots were then transferred to PGR-free RIM containing MS, 2.0% sucrose, 250 mg/l Cef and 75 mg/l Km. Rooting efficiency was 91% on Km-supplemented rooting medium.

Uniform wounding of the midrib resulted in a more efficient shoot regeneration (63% versus 16%) than the irregular, random wounding of lamina. Expression of StBEL5 fragment was detected in all randomly selected rooted transgene shoots analyzed by RT-PCR. StBEL5 is a marker gene coding for a transcription factor that enhances tuber formation in transgenic plants. This trait was used as a marker for stably transformed lines.

CONCLUSION

It is difficult to directly recommend any of the above presented protocols. They all serve their purpose enabling successful transformation of numerous potato cultivars and genotypes. Since there is a marked effect of the genotype perhaps the best approach is to find if some of the presented protocols are suitable for our potato cultivars or lines.

For non-applied transformation studies in molecular biology like carbohydrate metabolism the choice is obvious. The simple, singe step protocols of Rocha-Sosa *et al*. (1989) or Keil *et al*. (1989) are sufficient.

For other types of research it is perhaps better to choose some of the double-step (stage) protocols which can be quickly optimized to fulfill our needs. We had good experience with protocols of de Block (1988) and Wenzler *et al*. (1989), modifying them in accordance to recommendations and improvements from more recent protocols like those of Barell *et al*. (2002), Heeres *et al*. (2006) and Gustafson *et al*. (2006).

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