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RESEARCH

Comparison of DNA Walking Methods for Isolation of Transgene-Flanking Regions in GM Potato

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Abstract An important aspect in the safety assessment of transgenic plants is the exact location of transgene insertion sites within the host genome. However, robust standard operating procedures are not currently available. Using potato as a test species, different methodologies for the determination of insertion sites using a range of published protocols and commercially available kits were assessed in transgenic lines of varying degrees of complexity, from low copy number to complex re-transformed and co-transformed lines. Three commercial kits, APAgene^{TM} GOLD Genome Walking Kit (BIO S&T), DNA Walking SpeedUp^{TM} Kit II (Seegene), and Universal Vectorette^{TM} System (Sigma) were compared with an adaptor-mediated PCR technique. Overall, the APAgene^{TM} kit was used with a high success rate with low copy number potato lines, and also more complex co- and re-transformed lines, and adhering to key confirmation steps it was possible to obtain flanking sequence ranging in size from 300 to 2,500 bp and eliminate PCR artefacts from the analysis.

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Introduction

Methodologies for the production of genetically modified (GM) plants are continually being improved and crops containing multiple transgenes are widely cultivated [1–3]. Transformation can result in the integration of a variable number of transgene copies at different locations in the plant genome [4]. One aspect of the process that cannot at present be controlled is the exact location of the transgene insertion within the host genome which may have implications for transgene stability and potential for unintended effects on plant metabolism. For this reason, analysis of the transgene insertion site is an important component of any safety assessment process for a particular event. By determining the genomic regions flanking the transgene insertion it is possible, by sequence alignment with published mapping, Expressed Sequence Tag (EST) and sequence data, to determine the position of the transgene in the genome. It can also determine whether any functional or regulatory genes have been disrupted and whether any new open reading frames (ORFs) have been created, resulting in the possible synthesis of novel chimeric proteins. Comparative sequence analysis may not always be possible due to limited genomic databases for the crop species in question. However, knowledge of gene function will increase with time. It is also clear that not all functions and/or sequence patterns of plant genes and non-coding regions are known. Thus, flanking sequence information will not necessarily provide unequivocal evidence for safety but will support the risk assessment substantially.

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Knowledge of the flanking sequences also provides information on unique identification sequences that can be used to detect the transgenic event in question.

There are currently a number of PCR-based protocols [5] that have been used for 'walking' into uncloned plant DNA to isolate the genomic regions flanking transgenes as an alternative more rapid approach to the time-consuming procedure of screening genomic libraries. These methods include ligation-mediated or adaptor-mediated PCR (LM-PCR), inverse PCR (IPCR) and thermal asymmetric-interlaced PCR (TAIL-PCR). Each of these protocols has been used successfully in the large-scale isolation of transgene-flanking regions, for example LM-PCR [6], IPCR [7] and TAIL-PCR [8]. Although each of these methods have been employed effectively in different crop species containing particular types of transgene insertion, they can be difficult to optimise for a range of different insertions as they are hampered by steps in the protocols that are laborious and technically demanding. Limitations of these methods may include the successful annealing of degenerate primers, requirements for restriction sites, and efficiency of ligation reactions. This makes the routine and reproducible analysis of transgene insertion sites challenging and places a greater burden on both the applicant and the regulator in the risk assessment procedure. Additionally, quality control of the entire process is difficult as the protocols generally involve multiple steps, employing reagents and equipment from many different suppliers. Thus, there is still a requirement for stream-lining protocols and determining the key quality control parameters that must be addressed. Accordingly, this has led to the development of several commercial kits on the market for genome walking.

The primary aim of this research was, therefore, to develop standard, validated procedures for the isolation of transgene-flanking regions in one of the major GM crops, potato (*Solanum tuberosum* L.) containing a range of different transgene insertion patterns. Previously, the majority of applications for approval for commercial release have focused on single-event transformants, however, stacking of transgenes to produce plants with multiple beneficial traits is a trend that will become increasingly common [1]. Testing of methodologies that can routinely accommodate more complex, multiple integration sites were, therefore, also included. Three commercial DNA Walking Kits [APAgene^{TM} GOLD Genome Walking Kit (BIO S&T, Montreal, QC, Canada), DNA Walking SpeedUp^{TM} Kit II (Seegene Inc., Seoul, South Korea), Universal Vectorette^{TM} System (Sigma-Aldrich, Poole, UK)] were initially evaluated with an adaptor-mediated PCR technique as a study funded by the Food Standards Agency (FSA) in the UK (project G02002).

Materials and Methods

Transgenic Potato Lines

Solanum tuberosum L. (cv. Desiree) and *Solanum phureja* (cv. Mayan Gold) plants were transformed by *Agrobacterium tumefaciens* strain LBA4404 carrying derivatives of the binary vector pBIN19 with the neomycin-phosphotransferase II (*npt*II) gene (selective marker) using the method of Ducreux et al. [9] and contained: a potato terpene synthase (*TPS*) (Taylor et al. unpublished), the algal gene *β*-carotene ketolase (*BKT1*) or a bacterial gene encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS) [10]. Co-transformed potato lines contained a potato pectin methyl esterase (PME) gene (Taylor et al. unpublished), and re-transformed potato lines were generated by transforming lines expressing the *Erwinia uredovora* phytoene synthase gene *CRTB* [9] with a pGPTV-bar vector and potato antisense zeaxanthin epoxidase (ZEP) insert (Taylor et al. unpublished). Details of the transgenic lines used in this study are summarised in Table 1.

Potato Leaf DNA Extraction

Potato transgenic lines were grown under glasshouse conditions [9] and young leaf tissue was harvested from 4 to 5 week old plants for all lines tested to standardise the age of the material extracted, immediately frozen in liquid N_2, and stored at −80°C until processing. DNA was extracted from 100 mg samples of frozen leaf tissue using AquaGenomic^{TM} aqueous solution (Mo Bi Tec, Göttingen, Germany) following the manufacturer's instructions. The AquaGenomic^{TM} solution was the preferred high-yielding method due to its reliability and scalability in an assessment with six methods including commercial kits, proprietary reagents, and a CTAB method for the extraction of DNA from potato leaf (data not shown), but the applicability of the solution for other tissues and different species may require validation. The DNA was quantified using the Quant-iT^{TM} PicoGreen$^{®}$ dsDNA Assay kit, (Invitrogen Ltd., UK) according to the manufacturer's instructions, and diluted to 100 ng/μl in sterile-distilled water (SDW).

Evaluation of Several DNA Walking Methods for the Isolation of Transgene-Flanking Regions in Potato

Three commercial DNA Walking Kits [APAgene^{TM} GOLD Genome Walking Kit (BIO S&T, Canada), DNA Walking SpeedUp^{TM} Kit II (Seegene, South Korea), Universal Vectorette^{TM} System (Sigma-Aldrich, UK)] were initially evaluated with an adaptor-mediated PCR

Table 1 Summary of transgenic potato lines selected for obtaining genomic DNA flanking the transgene insertion site by various DNA walking methods

Potato transgenic line	Species	Cultivar	Construct(s) present	Copy number[a]
Single-event transformation				
Bkt1-1	*Solanum phureja*	Mayan Gold	pBIN19 (*bkt1*)	2
Bkt1-2	*Solanum phureja*	Mayan Gold	pBIN19 (*bkt1*)	1
CrtB-9	*Solanum tuberosum*	Desiree	pBIN19 (*crtB*)	1
Dxs-19	*Solanum tuberosum*	Desiree	pBIN19 (*dxs*)	1
Dxs-36	*Solanum tuberosum*	Desiree	pBIN19 (*dxs*)	2
TPS4	*Solanum tuberosum*	Desiree	pBIN19 (*tps*)	3
TPS6	*Solanum tuberosum*	Desiree	pBIN19 (*tps*)	1
TPS8	*Solanum tuberosum*	Desiree	pBIN19 (*tps*)	2
TPS9	*Solanum tuberosum*	Desiree	pBIN19 (*tps*)	5
TPS16	*Solanum tuberosum*	Desiree	pBIN19 (*tps*)	2
TPS42	*Solanum tuberosum*	Desiree	pBIN19 (*tps*)	2
TPS51	*Solanum tuberosum*	Desiree	pBIN19 (*tps*)	3
Co-transformed				
CT-3	*Solanum tuberosum*	Desiree	pBIN19 (*pme*)	3
CT-88	*Solanum tuberosum*	Desiree	pBIN19 (*pme*)	3
CT-184	*Solanum tuberosum*	Desiree	pBIN19 (*pme*)	2
Re-transformed				
B9Zep-4	*Solanum tuberosum*	Desiree	pBIN19 (*crtB*) + pGPTV (*zep*)	2
B9Zep-13	*Solanum tuberosum*	Desiree	pBIN19 (*crtB*) + pGPTV (*zep*)	1

[a] Copy number determined by Southern analysis and real-time PCR and was in agreement in all cases (see "Materials and methods" section)

technique with single copy, *Agrobacterium*-derived lines of transgenic potato (Table 1).

Primary PCR Test with Gene-Specific Primers to Ensure Successful Amplification

Gene-specific primers (GSP) were designed for both the right and left termini of the T-DNA borders of vector pBIN19 following each of the manufacturer's kit recommendations. Each GSP was tested in a PCR with a corresponding Test primer (LBT and RBT, Table 2) designed on the opposite strand of the sequence before the end of the left border (LB) and right border (RB) of pBIN19 to ensure successful amplification in each transgenic line during the DNA walking procedure. Standard PCR reactions were performed with 1 µl DNA (100 ng) in a 24 µl master mix consisting of: 1× Go Taq® Reaction Buffer (Promega, UK), 0.2 mM each dNTP (Promega, UK), 0.3 µM each primer (Eurogentec Ltd., UK) and 1.0 Unit Go Taq® DNA Polymerase (Promega, UK). PCR amplification was based on an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s, extension at 72°C for 30 s and a final elongation at 72°C for 7 min using a Veriti™ 96-Well Thermal Cycler (Applied Biosystems Ltd., Warrington, UK). PCR products (5 µl) were analysed on 1.0% agarose gels containing 0.5 µg/ml ethidium bromide in 0.5× TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA) buffer.

For adaptor-mediated PCR, the method described by Spertini et al. [11] was optimised for potato transgenic lines using the following modifications [12]. Genomic DNA (0.5 µg) was digested separately with 20 Units of *Bam*HI, *Eco*RI and *Hind*III, and the appropriate adaptors were used for these restriction enzymes. PCR was performed in a 25 µl total reaction volume using 2 µl ligated DNA, 1 µl 2.5 mM nested primer, 1 µl 2.5 mM AP1/AP2 [11] and 21 µl ABgene 1.1× ReddyMix™ (1.5 mM $MgCl_2$; ThermoFischer Scientific, UK). The second PCR amplification was performed using 2 µl of product from the first PCR amplification. Commercial DNA Walking kits were used following the manufacturer's instructions. In the case of the Universal Vectorette™ System (Sigma-Aldrich), Vectorette DNA libraries were generated by digesting target DNA with *Bam*HI, *Eco*RI and *Hind*III.

Pre-Screen Method Based on Real-Time PCR and the Universal Probe Library (UPL) for Determination of the Length of Vector Backbone

Due to a high frequency of backbone integration in transgenic potato lines, a pre-screen method based on real-time quantitative PCR and the Universal Probe Library (UPL,

Table 2 Oligonucleotide primers and probes used in this study

Name	Orientation	Sequence 5′–3′	Amplicon size (bp)[a]	Target
Real-time PCR				
Bin19*npt*F1	Forward	ccacagtcgatgaatccaga	61	Neomycin-phosphotransferase II (*npt*II)
Bin19*npt*R1	Reverse	atgcctgcttgccgaata		
UPL#31	Probe (forward)	ttccacca		
sucF1	Forward	tcgtttgaggcctggtgtct	91	Sucrose synthase (*suc*)
sucR1	Reverse	gaattgcaaatactcagggacaga		
sucP1	Probe (forward)	aatacatccgtgtgaacgtcaacgcactag		
Gene-specific primers[b]				
B2BioLF1	Left Border	ggctttccccgtcaagctctaaat		pBIN19
B2BioLF2	Left Border	ggctccctttagggttccgatttag		pBIN19
B2BioLF3	Left Border	ttagtgctttacggcacctcgacc		pBIN19
RBBioRF1	Right Border	gtgaccttaggcgacttttgaacgc		pBIN19
RBBioRF2	Right Border	cgcgcaataatggtttctgacgtat		pBIN19
RBBioRF3	Right Border	ctccttcaacgttgcggttctgtc		pBIN19
Insert-size PCR				
M13F	Forward	gtaaaacgacggccagt		pGEM®-T Easy Vector
M13R	Reverse	ggaaacagctatgaccatg		
PCR confirmation				
LBT1	Forward	tggccctgagagagttgcag		pBIN19
LBT2	Forward	ttcaccagtgagacgggcaac		pBIN19
RBT1	Reverse	gcgggacaagccgttttac		pBIN19
RBT2	Reverse	ctgaaggcgggaaacgacaatc		pBIN19

[a] In some cases amplicon size was dependent upon the corresponding gene-specific primers used during PCR or the cloned insert tested for each transgenic line under study

[b] Gene-specific primers listed were designed for use with the APAgene™ GOLD Genome Walking Kit (BIO S&T, Montreal, QC, Canada)

Roche Diagnostics Ltd, Burgess Hill, UK) for determination of the length of vector backbone was developed. Prescreening for vector backbone was used with single copy transgenic lines in order to identify a starting point for a DNA Walking procedure and, therefore, reduce time and costs. A series of 19 assays with probes were designed to generate products of around 500 bp using the UPL Assay Design Center and recommended parameters from Roche (https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp) to cover 90% of the pBin19 vector backbone from both the left (4,539 bp) and right borders (3,367 bp).

Real-Time PCR

PCR reactions were performed with 1 µl undiluted DNA and 24 µl FastStart TaqMan®Probe Master (Rox) mix and run on the automated ABI 7500 Fast Real-Time PCR System (Applied Biosystems, UK) using a standard 7500 run mode and three-step cycle: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min and 72°C for 30 s. Primers were included at a final concentration of 900 nM per reaction, and UPL probes used at 100 nM. A positive signal generated from a set of primers and probe indicated the presence of a particular segment of vector backbone in a transgenic line, whereas no signal indicated the absence of backbone. A set of gene-specific primers were subsequently designed near the end point of the vector backbone in each line (if present) in order to start the DNA Walking procedure.

Transgene Copy Number Determination

The comparative threshold cycle (ΔΔCt) method of relative quantification by duplex PCR was used to determine the number of copies of the target gene on two independent occasions normalised to the endogenous control and relative to a reference sample (calibrator) with a known copy number by the amount of target $= 2^{-\Delta\Delta Ct}$ (User Bulletin #2, Applied Biosystems, UK). The copy number of the same transgenic lines was also determined and compared to Southern analysis as detailed previously [10], and the numbers were in agreement in all cases. The antibiotic marker gene *npt*II located between the LB and RB

sequences on vector pBIN19 was selected as the target for transgenic lines and a real-time PCR assay with UPL probe was designed using the UPL Assay Design Center (Roche, UK). The potato sucrose synthase (*suc*) gene (Genbank accession number U21129) was chosen for the design of an endogenous reference assay to standardise the amount of potato DNA added to a reaction (single copy gene; [13]), and a set of primers with probe were designed using the Primer Express Software® Version 3 (Applied Biosystems, UK) (Table 2). The *suc* gene probe was labeled at the 5′ end with the reporter dye Yakima Yellow® (Eurogentec Ltd., UK) and modified at the 3′ end with a Black Hole Quencher™ 1 (BHQ-1™; Eurogentec Ltd., UK). Reactions were run on the automated ABI 7500 Fast Real-Time PCR System (Applied Biosystems, UK) using a standard 7500 run mode and two-step cycle: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Both *npt*II target and *suc* reference gene primer sets were included at a final concentration of 900 nM per reaction and probes used at 100 nM.

Long PCR with an Anchor Primer for Determining the Orientation of Vector Backbone as a Result of Integration

When real-time PCR results indicated that a large portion of the cloning vector backbone (pBIN19) was integrated in a particular transgenic line, a long PCR with an anchor primer was used to determine whether the orientation of this integration was from the left or right border. The Bin19*npt*F1 and Bin19*npt*R1 primers (Table 2) were used as anchor primers and matched with the series of primers used with the UPL probes in a conventional PCR going from both the left and right borders. PCR amplification was based on the standard conditions using 1.0 µl of DNA (100 ng) with 1.0 Unit of Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen, UK) and annealing set at 59°C for 30 s and extension at 68°C for 3 min. The presence of product with either series of primers going from the left or right border indicates the orientation of the transgene integration. A set of GSPs were subsequently designed from the correct orientation near the end point of the vector backbone in each line (if present) in order to start the DNA Walking procedure.

Downstream Processing of PCR Products Following DNA Walking

If a PCR was successful, depending on the number of distinct DNA bands visualised following gel electrophoresis for each sample, downstream processing involved one of the following steps to purify PCR products for sequencing. Single PCR products were directly purified using ExoSAP-IT® (USB Europe GmbH., Staufen, Germany) following the manufacturer's instructions. If up to three distinct PCR products were detected, DNA was extracted from agarose gels using the Wizard® SV Gel Clean-Up System (Promega, UK) according to the manufacturer's instructions prior to cloning and electrotransformation. In both cases, a nested PCR confirmation stage (described below) using 1 µl of purified product was performed to exclude artefact bands prior to cloning and sequencing.

In the detection of multiple PCR products, purification involved using the Wizard® SV Gel Clean-Up System (Promega, UK) according to manufacturer's instructions, followed by the cloning of products (1.5 µl) into the pGem-T® Easy Vector System I kit (Promega, UK) according to the manufacturer's instructions. Cloned products were subsequently electrotransformed into ElectroMAX™ DH510B™ *E. coli* cells (Promega, UK) using a Micro-Pulser™ (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) set at 1.8 kV with 0.1 cm cuvettes using a 1 µl volume of ligation reaction mixed with 10 µl ElectroMAX™ DH510B™ cells according to the manufacturer's instructions. Transformed colonies were isolated on Luria broth (LB) plates containing 50 µg/ml ampicillin, 0.5 mM IPTG and 80 µg/ml X-gal after incubation at 37°C for 16 h. For each transformation event, a total of 10–20 white colonies were picked with a pipette tip from LB plates and suspended in 20 µl SDW in the wells of a 96-well PCR plate on ice. The number of colonies screened can be reduced or increased depending on the number of PCR products generated during DNA Walking PCR. A 2 µl volume of this colony suspension was used in a standard PCR with M13 Forward and Reverse primers (0.3 µM each) (Table 2) in a 23 µl master mix with annealing conditions at 55°C for 30 s and extension at 72°C for 1 min over 30 cycles. If a product of the correct size was detected, a nested PCR confirmation assay was performed immediately to confirm the absence of PCR artefact inserts before subsequent growth of transformants for plasmid preparations. Confirmation test primers were designed (LBT and RBT, Table 2) on the opposite strand to internal regions before the start of both the LB and RB of the T-DNA insertion for use with GSP primers. A 1 µl volume of purified PCR product or products generated with the M13 primers was added to a 24 µl master mix and amplified during standard PCR conditions over 25 cycles and PCR products (5 µl) were analysed by electrophoresis on 2% agarose gels as described previously. For every positive nested PCR confirmation test, 10 µl of the remaining colony suspension was inoculated into 5 ml LB medium containing ampicillin (50 µg/µl) and samples were incubated overnight for 16 h at 37°C with shaking (225 rpm). Plasmid DNA was extracted from 2 ml cell suspensions

using the QIAprep Spin Miniprep Kit (Qiagen, UK) according to manufacturer's instructions.

Sequencing PCR

Both strands of each PCR product or plasmid preparation (50–150 ng/µl) were sequenced using primers M13F and M13R, an automated ABI 3700 capillary sequencer (Applied Biosystems, UK) together with the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit according to the manufacturer's instructions (Applied Biosystems, UK).

Analysis of DNA Sequences

Sequence data obtained from transgenic lines were aligned with the vector pBIN19 sequence using the ClustalW2 multiple sequence alignment program (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and analysed for related sequences by a BLASTn program search of the non-redundant nucleotide (nr/nt) databases against the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/).

PCR Confirmation of Junctions

Following the analysis of sequences and identification of junctions in transgenic lines, additional primers were designed following the guidelines and conditions described previously to amplify the sequence starting from the T-DNA before the junction and crossing into the genomic DNA. PCR products were purified with ExoSAP-IT® (USB Europe GmbH., Germany) and sequenced directly to confirm the isolation of the junction.

Results and Discussion

Evaluation of DNA Walking Methods for the Isolation of Transgene-Flanking Sequences in Single Copy Lines of Potato

A pre-screen method based on real-time PCR and the Universal Probe Library (Roche, UK) was initially used for determination of the sequence length of vector backbone in the transgenic potato lines under study. The single copy lines, Dxs-19 (*S. tuberosum*) had LB vector backbone up to ~2.7 kb integrated, but no RB backbone was present, whereas Bkt1-2 (*S. phureja*) and CrtB-9 (*S. tuberosum*) appeared to have the total cloning vector integrated, and the orientation of this integration using an anchor primer was from the RB. This pre-screening for vector backbone served to identify a starting point for a DNA Walking procedure. It has been hypothesised that the LB acts primarily to terminate the 5′–3′ T-strand processing initiated by the VirD2 product at the RB and that failure to recognise the LB sequence can sometimes cause over-run of the T-strand into vector backbone DNA during T-DNA transfer [14].

Gene-specific primers were subsequently designed for each protocol to determine flanking regions from both the LB and RB and three commercial DNA Walking Kits (APAgene™ GOLD Genome Walking Kit, DNA Walking SpeedUp™ Kit II and Universal Vectorette™ System) were compared with the adaptor-mediated PCR technique in terms of success and reliability. In terms of success rate, both the APAgene™ and SpeedUp™ kits were superior and comparable in that five out of six flanking regions were determined from these three transgenic lines (Table 3). In one example for the LB of Bkt1-2, four and two independent bands tested produced the same flanking regions using the different APAgene™ and SpeedUp™ kit primers, respectively (Figs. 1, 2), and indicated a truncation to all of the T-DNA left border and *lac*I sequences, and part of the M13 *ori* sequence of pBIN19. The same flanking regions determined for all three transgenic potato lines were in agreement using both these commercial kits. All junctions were subsequently confirmed following PCR confirmation and sequencing (data not shown).

Both the adaptor-mediated PCR and Vectorette™ System are similar methods in principle but produced less consistent results and were more time consuming due to the need for DNA digestion with restriction enzymes and ligation steps in addition to two rounds of PCR (Table 3). Only one PCR product was generated using adaptor-mediated PCR from the LB of line Bkt1-2, but the junction was successfully determined by sequencing and this was in agreement with the APAgene™ and SpeedUp™ kits (Table 3). Although many products were amplified using the Vectorette System™, vector sequence was only reached for one product from the LB of line CrtB-9, while the remaining bands isolated were identified as artefacts generated by the kit primer.

Transgene-Flanking Regions of Single Copy Potato Transgenic Lines

A BLASTn program search of the databases at the NCBI revealed that the most probable related genomic regions flanking the transgene insertion sites of *S. tuberosum* lines CrtB-9 and Dxs-19 were of *Solanum* origin (Table 4). However, in the case of *S. phureja* line Bkt1-2, the most significant alignments revealed that the transgene had inserted between sequences related to bacterial antibiotic resistance plasmid/transposon-like sequences; PCR verification confirmed all these genomic sequences as non-host genomic DNA nor vector-derived material. Interestingly,

Table 3 Results summary of DNA walking methods for single copy potato transgenic lines

	Junctions detected in potato transgenic lines (single copy)					
	Bkt1-2 (*Solanum phureja*)		CrtB-9 (*Solanum tuberosum*)		Dxs-19 (*Solanum tuberosum*)	
Methods:	LB	RB	LB	RB	LB	RB
APAgene™ GOLD Genome Walking Kit (BIO S&T)	+ (4)[a]	+ (1)	+ (1)	+ (1)	+ (2)	V (2)
DNA Walking SpeedUp™ Kit II (Seegene)	+ (2)	V (1)	+ (1)	+ (1)	+ (2)	+ (2)
Universal Vectorette™ System (Sigma-Aldrich)	–	–	V (1)	–	–	–
Adaptor-mediated PCR	+ (1)	–	–	–	–	–

[a] Numbers in parenthesis indicate the number of different size products in which sequence information was obtained using the method

Key: *LB* Left border, *RB* Right border, + = Junction identified, − = No products or PCR artefacts, *V* Vector backbone detected, further walking required

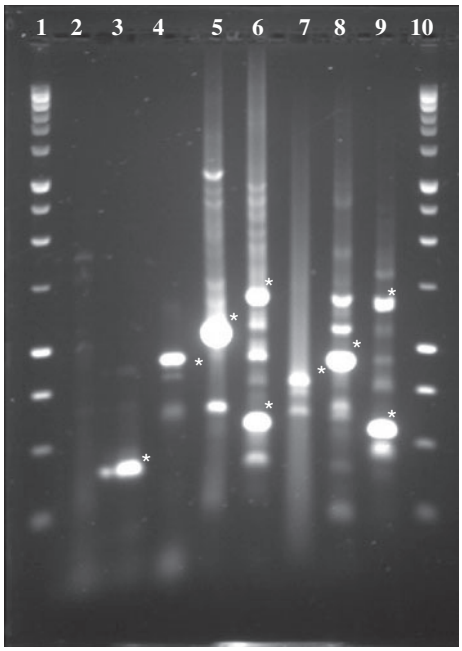

Fig. 1 Amplification of T-DNA left border flanking regions from a single copy line of transgenic potato (Bkt1-2) using the DNA Walking SpeedUp™ Kit II (Seegene) and APAgene™ GOLD Genome Walking Kit (BIO S&T); 5 µl of PCR products loaded per lane. *Lane 1* 1 kb DNA ladder, 250–10,000 bp; Final round nested PCR products: *lanes 2-5* ACP1 to ACP4 SpeedUp™ primers, respectively; *lanes 6–9* DRTA to DRTD APAgene™ primers, respectively; lane 10, 1 kb DNA ladder. * All bands excised and purified using Wizard® Gel Clean-Up System (Promega) for direct sequencing

the same bacterial-like sequences were also identified by the BLASTn search at one of the left and right borders of the two copy line, *S. phureja* Bkt1-1 (Table 5). The origin of these bacterial-like sequences is unknown and we can only speculate without further DNA walking and sequencing from these flanking regions. A possible explanation for the presence of prokaryotic sequences in transgenic potato lines could be due to the incidental transposition of mobile genetic elements accompanying cloning steps [15]. It was also reported by the same authors that many published eukaryotic sequences contained the flanking inverted repeats of transposon Tn*10*, IS*10* and other prokaryotic IS elements. Bogosian et al. [16] reported residual copies of IS*10* in the chromosome of *E. coli* LBB84 after the precursor strain had been cured of Tn*10*, and that IS*10* transposed in *trans* from the chromosome to some recombinant plasmids grown in this host strain.

Evaluation of DNA Walking Methods for the Isolation of Transgene-Flanking Regions in Multicopy Lines of Potato

The next stage of this study was to test each DNA Walking method for locating the flanking regions in more challenging transgenic lines containing multiple T-DNA inserts, including co- and re-transformed lines. In an initial test with potato lines containing two transgene copies (e.g., Bkt1-1, Dxs-36, TPS6, TPS8, TPS16 and TPS42), the APAgene™ kit was more successful in determining the junctions as the SpeedUp™ method consistently generated artefact bands. This is highlighted in one example for determining the junction from the LB of lines TPS6 and TPS8 (Fig. 3a, b). Although many PCR products were amplified using both kits, the use of a nested PCR confirmation test and subsequent sequencing demonstrated that all bands isolated by gel extraction and cloning with the SpeedUp™ kit were artefacts generated by the UniP2 primer. In contrast, all bands isolated and sequenced from these six transgenic lines with the APAgene™ kit resulted in the determination of transgene-flanking regions, and this was confirmed by analysing several independent bands in each case (Table 5). The successful application of the protocol involving cloning and PCR verification of insert size and PCR confirmation to exclude artefact bands for lines TPS6 and TPS8 is shown in Fig. 4a and b.

No flanking regions were located in any of the two or three copy potato lines (Table 5) using either the

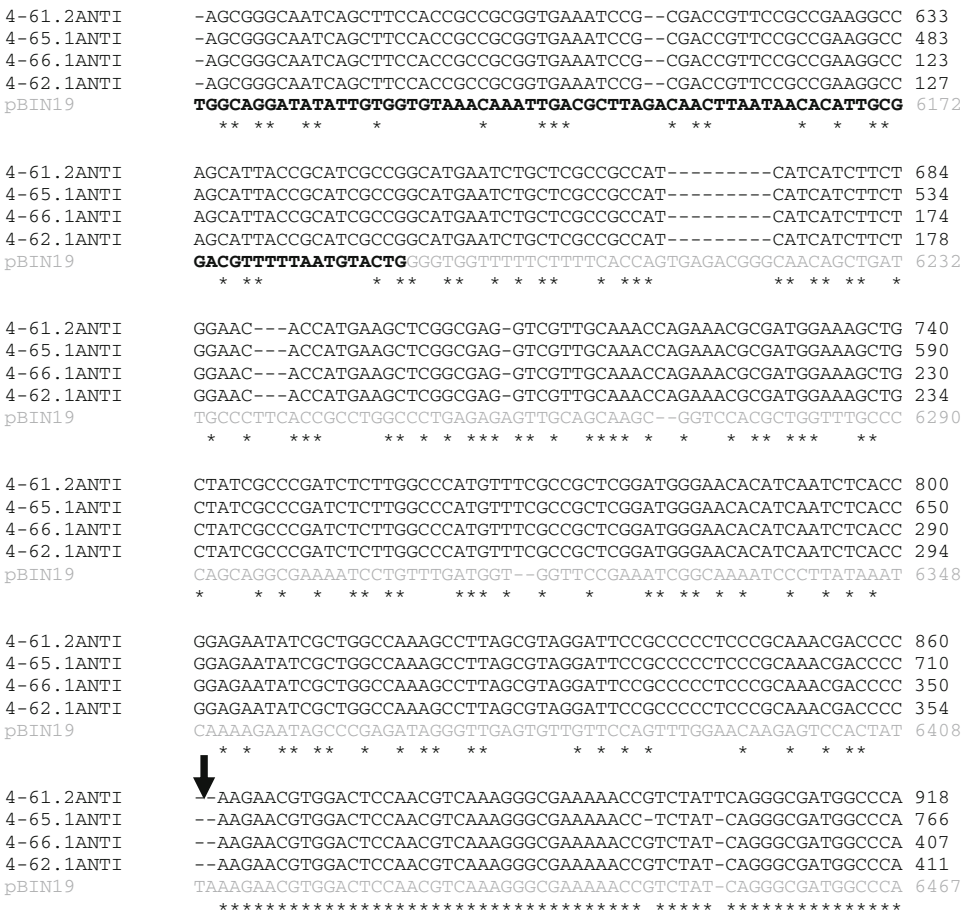

Fig. 2 Nucleotide sequence of four independent products generated from the left border flanking region of line Bkt1-2 using the APAgene™ GOLD Genome Walking Kit (BIO S&T). The *arrow* indicates the location of the junction in Bkt1-2 after alignment of sequences with vector pBIN19. *Letters* in *bold* indicate position of the left border of pBIN19. *identical bases within in a column of the aligned portion of the sequence

Table 4 Most significant alignments for the transgene-flanking regions of single copy potato transgenic lines based on BLASTn searches of the NCBI databases

Line[a]		BLASTn results of NCBI nucleotide databases		
		Accession number	*E* value	Alignment
Bkt1-2	LB	AF313472.2	0.0	*Pseudomonas aeruginosa* plasmid RPL11 transposon *Tn*1403.
	RB	AM992204.1	4e−37	*Psychrobacter psychrophilus* transposon *Tn*5080.
CrtB-9	LB	AC151957.1	6e−89	*Solanum tuberosum* chromosome 5 clone PGEC13.
	RB	EU124732.1	6.3	*Solanum lycopersicum* chromosome 3 clone C03HBa0030O03.
Dxs-19	LB	AC232052.1	5e−71	*Solanum tuberosum* chromosome 5 clone RH072I03.
	RB	AK325770.1	3e−125	*Solanum lycopersicum* cDNA, clone LEFL2025I06.

[a] Data for the most significant alignment was selected from those sequences generated from the largest PCR products recovered by DNA Walking using the APAgene™ (BIO S&T, Canada) and SpeedUp™ (Seegene, South Korea) kits

Key: *LB* Left border, *RB* Right border

adaptor-mediated PCR or Vectorette™ System and only artefact products were generated. It is clear that both of these methods would require further optimisation for determining the flanking regions in transgenic potato lines. The main disadvantages of such methods are: the presence of suitable restriction enzyme sites, successful ligation of the specific adaptors to restriction fragments obtained from genomic DNA, and the recommendation of an initial concentration of 500–1000 ng genomic DNA to perform the digestion step. In contrast, only ~100 ng DNA is required for the first PCR step with the APAgene™ and SpeedUp™ kits. In order to become useful for large-scale screening of transgenic plants, these procedures would have to be optimised and streamlined to allow fast and cheap analysis of many samples.

The APAgene™ kit had a high success rate and located both of the flanking regions at the LB and RB of Bkt1-1 and TPS8, both junctions at the LB of TPS42 and RB of

Table 5 Results summary for transgene-flanking regions of multi-copy potato transgenic lines using the APAgene™ GOLD Genome Walking Kit (BIO S&T, Canada)

Transgenic potato lines	Copy number[a]	Junctions isolated (number of products analysed per junction)[b]	
		Left border	Right border
Single transformation			
Bkt1-1[c]	2	2 (2[d]:1)	2 (6[d]:1)
Dxs-36[c]	2	ND	2 (7[d]:1)
TPS6[c]	2	1 (13)	2 (5[d]:1)
TPS8[c]	2	2 (8:3)	2 (2:1)
TPS16[c]	2	ND	1 (11)
TPS42[c]	2	2 (11:3)	ND
TPS4[c]	3	3 (5v:4:2)	ND
TPS51[c]	3	3 (6:1:1)	ND
TPS9	5	3 (1:3:5)	ND
Co-transformed			
CT-184	2	4 (4:6:3:1v)	2 (1:10)
CT-3	3	4 (7:1:3:1v)	2 (1:5)
CT-88	3	3 (5:3:2v)	3 (11:3:1v)
Re-transformed			
B9ZEP-13	1	3 (2:1v:2)	1 (1)
B9ZEP-4	2	2 (2:5)	3 (2v:1:3)

[a] Copy number determined by Southern analysis and real-time PCR (see "Materials and methods" section)

[b] Number of independent PCR products isolated and sequenced that identified the same junction, e.g., 2 different junctions identified (8:3) by 8 and 2 different sized products

[c] No flanking regions were isolated in any of the two or three copy lines tested by the Universal Vectorette™ System (Sigma-Aldrich, UK) or adaptor-mediated PCR method

[d] The same flanking regions were obtained using the SpeedUp™ (Seegene, South Korea) kit but no others were isolated at the LB or RB in the remaining two or three copy lines tested

ND Not determined, *v* vector backbone detected, further DNA walking required

Dxs-36 and TPS6, all three junctions at the LB of lines TPS4 and TPS51(Table 5). However, it was only possible to isolate one of the two flanking regions at the LB of TPS6 and one at the RB of TPS16, and three out of the five flanking regions at the LB of TPS9 (Table 5) with the APAgene™ kit. This failure to isolate all flanking regions could be explained by rearrangements in some of the T-DNA LB and RB regions selected for the design of gene-specific primers due to the integration process, leading to the loss of the sequences specific to the primers. Further DNA Walking PCR and screening of additional transformant colonies (25 each in total) did not result in the isolation of the remaining junctions in these transgenic lines. Although the SpeedUp™ kit was used successfully to locate one of the flanking regions from both the LB and RB of Bkt1-1, and one junction each from the RB of lines

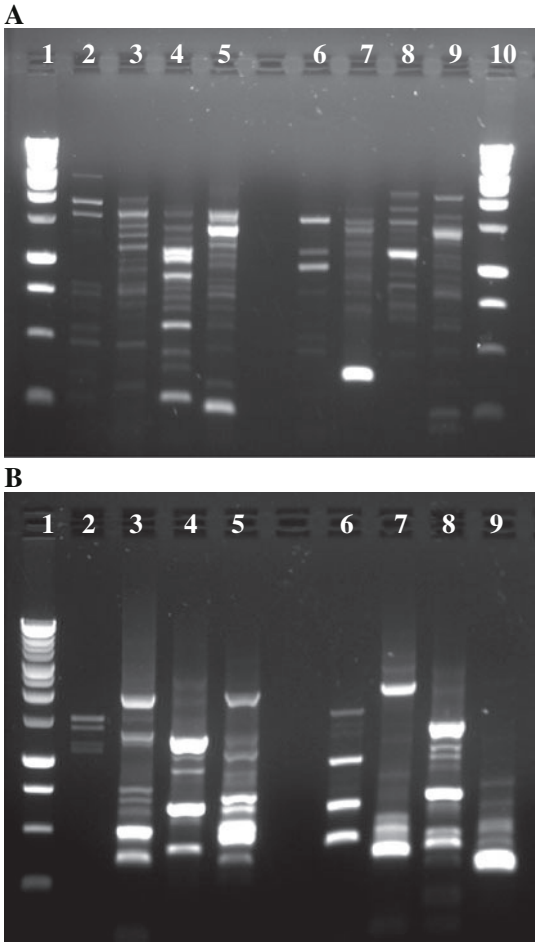

Fig. 3 Final-round PCR amplification products generated from the left border of two copy transgenic lines of potato (TPS6 and TPS8) using the **a** DNA Walking SpeedUp™ Kit (Seegene) and **b** APAgene™ GOLD kits (BIO S&T); 5 µl of PCR products loaded per lane. **a** *Lanes 1 and 10*, 1 kb DNA ladder, 250–10,000 bp; Final round nested PCR products: *lanes 2–5* ACP1 to ACP4 SpeedUp™ primers with TPS6, respectively; *lanes 6–9* ACP1 to ACP4 SpeedUp™ primers with TPS8, respectively. **b** *Lane 1* 1 kb DNA ladder, 250–10,000 bp; *lanes 2–5* DRTA to DRTD APAgene™ primers with TPS6, respectively; *lanes 6–9* DRTA to DRTD APAgene™ primers with TPS8, respectively

Dxs-36 and TPS6, none were located in the three copy lines, the majority of the bands analysed were artefacts. Changing the annealing temperatures and length of extension at 72°C, number of cycles during each PCR step, and altering the ratios of the kit primers (DW2-ACPN and/or UniP2) to gene-specific primers did not reduce the number of artefact bands generated using the SpeedUp™ kit (data not shown). For this reason, the APAgene™ kit was subsequently used in testing other potato transgenic lines with multiple insert copies (Table 5).

The presence of vector backbone DNA sequences in transgene loci was detected at both left and right borders following DNA Walking, and in the case of the re- and co-transformed potato lines, there were several examples of

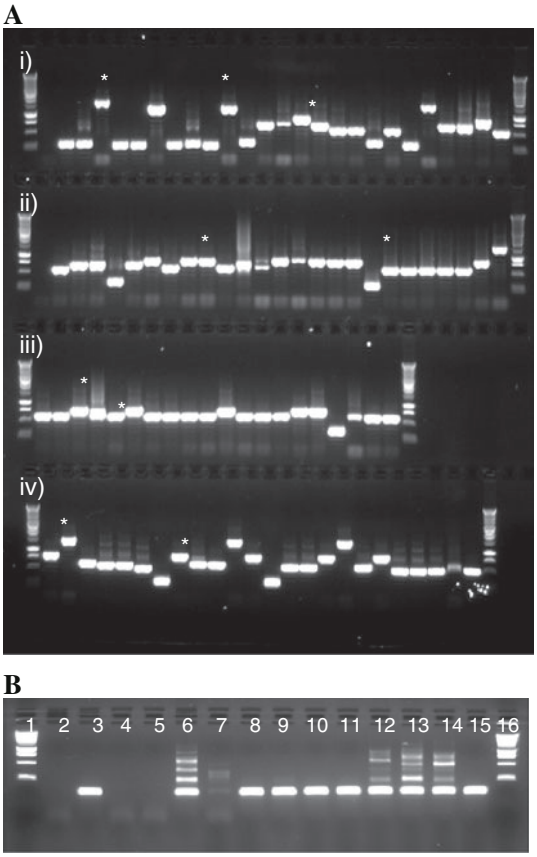

Fig. 4 Example of the procedures used for downstream processing of PCR products using the APAgene^{TM} GOLD kit (BIO S&T) for the left border (LB) of two copy transgenic lines of potato (TPS6 and TPS8); 5 µl of PCR products loaded per lane. **a** Colony PCR confirmation of cloned inserts obtained by electroporation using M13 forward and reverse primers from the LB of TPS6 (i and ii) and TPS8 (iii and iv). **b** Nested PCR screen of some of the above M13 products* to exclude artefacts using the confirmation test primer LBT1; a 1 µl volume of the M13 PCR product was re-amplified. *Lanes 1 and 16* 1 kb DNA ladder, 250–10,000 bp; *lanes 2–3* negative (1 µl SDW) and positive (1 µl pBIN19 at 1 ng/µl) controls for confirmation PCR; *lanes 4–15* show results using nested PCR following the amplification of (1 µl) first-round M13 products; *lanes 4–5*, negative controls (SDW); *lane 6*, positive control (pBIN19); *lanes 7–15*, a range of different cloned inserts from lines TPS6 and TPS8. *Indicates M13 PCR products used in nested PCR confirmation step

more flanking regions being isolated than the copy number estimation suggested were present (Table 5). An example of this is highlighted with the single copy (*zep* gene) re-transformed line B9ZEP-13 in which three flanking regions/or backbone were isolated from the LB, whereas only one junction was obtained at the RB (Table 5). In the remaining re-transformed two copy (*zep* gene) line B9ZEP-4 tested, three flanking regions/or backbone were detected at the RB and two junctions at the LB. The same pattern emerged with co-transformed two and three copy potato lines CT-184 and CT-3 tested, respectively, as four flanking sequences/or backbone were isolated at the LB in each (Table 5). In contrast, three flanking sequences/or backbone each were isolated at the LB and RB in line CT-88, in agreement with the copy number determination. Following the analysis of sequences, a PCR assay was designed to amplify a sequence crossing over the junction into the genomic DNA/or backbone to confirm the identification of flanking regions/or backbone, and this was positive in all the cases described (data not shown). Several explanations for the discrepancy between the copy number determination and the number of flanking regions detected in several transgenic lines can be offered. Firstly, the inherent inaccuracies of Southern analysis and the possibility of DNA rearrangements in the *npt*II gene sequence used for real-time PCR could account for an underestimation of copy number. Secondly, the integration of vector backbone without a complete T-DNA sequence (*npt*II gene deleted) could also account for the discrepancy. The demonstration of such a discrepancy in these complicated transgenic potato lines with experimental data by additional DNA Walking and sequencing was outside the scope of this research.

Overall, the APAgene^{TM} kit and the employment of key confirmatory tests were used with a high success rate with potato lines containing 1–3 transgene copies in the isolation of flanking regions/and or identification of vector backbone and it was possible to obtain products ranging in size from 300 to 2,500 bp (Figs. 1, 3). It is probable that additional screening of PCR products can be employed to successfully isolate flanking regions in potato lines containing more than three transgene copies. The reasons for the greater success rate of this kit over the Seegene kit may be linked to the initial PCR step with a gene-specific primer to generate single strand DNA fragments, thus increasing the concentration of potential flanking sequences before the addition of degenerate random tagging (DRT) kit primers to form double strand DNA products. The addition of a DRT-primer digestion reaction to eliminate non-specific background caused by both free and incorporated DRT primers may have also prevented the generation of artefact products, which was an inherent problem using the SpeedUp^{TM} kit with potato lines.

Examples of DNA Rearrangements Detected in the Vector Backbone of Potato Transgenic Lines

The application of the DNA walking methodology was not only successful for locating the transgene-flanking regions but was also useful in the detection of additional DNA rearrangements in the vector backbone of some potato transgenic lines. In one example for a re-transformed line (B9ZEP-4), there was a truncation to the T-DNA right border sequence and immediately downstream from this, two separate rearrangements in the pBIN19 vector

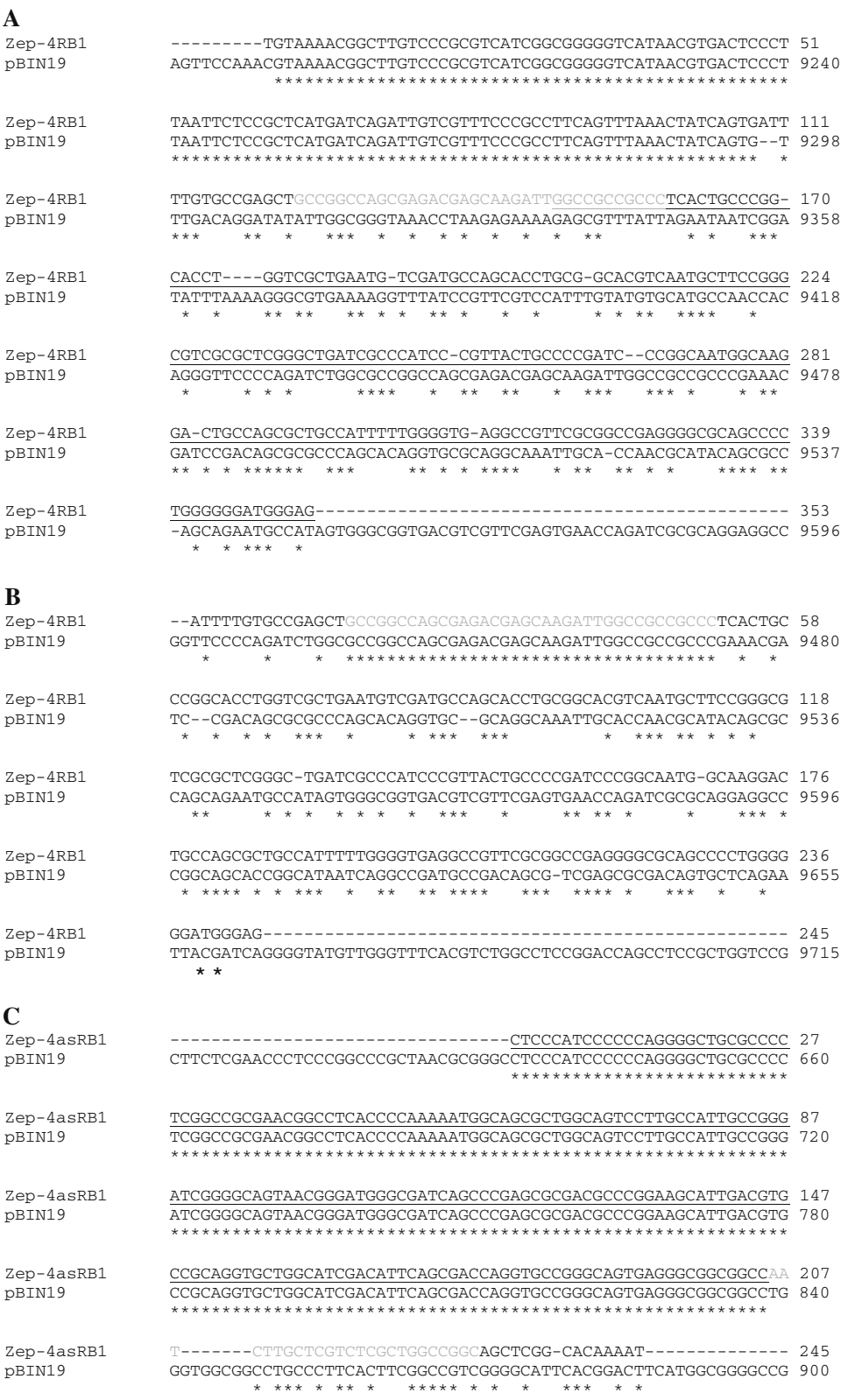

Fig. 5 Nucleotide sequence isolated from the right border of line B9ZEP-4 showing DNA rearrangements in the vector backbone of pBIN19. **a** The alignment of the total 353 bp of sequence isolated using the APAgene^{TM} GOLD kit (BIO S&T) with pBIN19; 100% homology (108 bp) to part of the Nos promoter and the T-DNA right border (nucleotides 9,191–9,297). **b** A short stretch of vector backbone sequence (36 bp) in light grey font showing 100% homology to part of the tetA gene of pBIN19 (nucleotides 9,438–9,473). **c** A stretch of vector backbone of 205 bp (underlined font) inserted in reverse complement showing 100% homology to part of the kilA gene of pBIN19 (nucleotides 634–838). *identical bases within in a column of the aligned portion of the sequence

sequence (Fig. 5a–c); a short piece of vector backbone sequence (36 bp) with 100% homology to part of the *tet*A gene (nucleotides 9,438–9,473) was followed by 205 bp of backbone inserted in reverse complement and showing 100% homology to part of the *kil*A gene of pBIN19 (nucleotides 634–838).

One reason for the common occurrence of vector backbone sequence integration in our potato transgenic lines could be due to the genotype of the transforming *A. tumefaciens* strain employed, in our case *A. tumefaciens* LBA4404. It was previously reported that the bacterial strain had an influence during the transformation of potato, and LBA4404 was more prone to deliver and integrate backbone sequence either at the left or the right border compared to AGL1 [17]. Strain LBA4404 also had a tendency to facilitate full backbone integration, in addition to only backbone integration in T-DNA negative plants more efficiently than AGL1. Evidence of backbone integration has been reported from several plant species: tobacco [18], rice [19], *Arabidopsis* [20], strawberry [21], barley [22], including potato [23]. An initial assessment of potential backbone integration was done on 3 transgenic Karnico lines [23] and showed a complex pattern of repeat T-DNA and backbone sequence integration often separated by potato DNA.

An understanding of the transgene insertion site within the host genome is an essential first step toward understanding the importance of the transgene insertion position and in aiding the safety assessment process for a particular transgenic event. However, the transfer of vector backbone sequences into plants also has a number of practical implications. The erroneous integration of vector backbone sequence might have unpredictable effects such as causing mutations or the silencing of endogenous or non-endogenous gene(s) [24, 25]. In addition, governmental regulatory guidelines may require a description of DNA sequences transferred to a transgenic plant, and, therefore, screening for the possible transfer of vector backbone sequences must be carried out as a requirement for gaining regulatory approval for environmental release of genetically modified crops.

Several key quality control parameters were identified during the development of a standard operating procedure for the isolation of transgene-flanking regions in potato. Firstly, a robust method of DNA extraction is required to yield DNA of a sufficient purity and decent yield for downstream analysis. The AquaGenomic^{TM} solution was the preferred high-yielding method due to its speed, reproducibility and scalability. Secondly, several important confirmatory steps including, successful PCR verification of all gene-specific primers designed, pre-screening for the length of vector backbone (if necessary) and PCR with an anchor primer to determine the orientation of integrated vector backbone prior to starting DNA Walking will reduce time and cost. Thirdly, the cloning of PCR products and screening of transformants by colony PCR to confirm insert sizes and a subsequent nested confirmation PCR is essential to avoid the downstream processing of PCR artefacts. Not all of these steps may be necessary for the routine and reproducible analysis of transgene insertion sites but following these key steps with the APAgene^{TM} kit should ensure the successful isolation of transgene-flanking regions in potato. However, although methods for the isolation of transgene junction sequences may be species dependent and the applicability of the method will require investigation, the use of the same confirmatory tests will ensure that the problem of PCR artefacts can be identified and removed from analyses.

Conclusions

In this study, we have demonstrated that a DNA walking PCR-based approach could be reliably used to not only identify the T-DNA insertion site in the potato genome but also used to investigate the frequency of backbone sequence integration in populations of transgenic potato. We have developed a strategy for the reliable identification of genomic flanking sequences of T-DNA insertions involving the APAgene^{TM} commercial kit which can be streamlined to avoid PCR artefacts, and the protocol allowed us to recover considerably more junction sequences compared to the other methods evaluated. The robustness and reliability of this method is demonstrated by the identification of both LB and RB flanking sequences in multicopy transgenic potato lines derived from single transformation, co- and re-transformation events, and thus can be considered as a valid alternative to existing genome walking protocols.

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