Duplex, triplex and quadruplex PCR for molecular characterization of genetically modified potato with better protein quality

Potato (*Solanum tuberosum* L.) is the most important tuberous vegetable crop of the world. The essential amino acids such as lysine, tyrosine and sulphur-containing amino acids, i.e. methionine and cysteine limit the nutritive value of potato protein[1]. AmA1 is a seed-storage protein with exceptionally high levels of all essential amino acids. For nutritional enhancement using genetic engineering tools, the gene encoding this protein can compensate for amino acid deficiencies of the seed proteins of the target crops. The gene encoding amaranth seed albumin (AmA1) from *Amaranthus hypochondriacus* has been cloned[2] and a patent has also been granted[3]. In 2000, Chakraborty *et al.*[4] successfully introduced the *AmA1* gene with the tissue-specific granule-bound starch synthase promoter and constitutive *CaMV* 35S promoter in potato.

As the global area for genetically modified (GM) crops has touched 125 million ha by 2008 (ref. 5), there is an urgent need to develop precise and reliable GM detection methods to meet the mandatory regulatory obligations. The Supreme Court of India has stipulated that prior to conducting field trials of GM crops, a protocol for testing contamination up to 0.01% has to be established[6]. Qualitative and quantitative analytical methods are required for monitoring of GM crops during their limited and large-scale field trials, even after their commercialization and for post-release monitoring. PCR is the most widely used analytical method for both qualitative as well as quantitative detection of GM crops. The qualitative multiplex PCR methods have been developed to detect three lines of GM potato with *cry3A* gene[7]. For diagnosis of vegetable GM crops under confined field trials in India, multiplex PCR assays have been developed for simultaneous detection of *cry1Ac* gene, *CaMV* 35S promoter and *SRK* gene in *Bt* cauliflower[8]; *osmotin* gene, *CaMV* 35S promoter and endogenous *LAT52* gene in GM tomato[9] and *cry1Ab* gene, *CaMV* 35S promoter, *nptII* marker gene and endogenous *UGPase* gene in *Bt* potato[10]. Recently, a multiplex PCR method simultaneously detecting six commonly used marker genes has been developed as an efficient tool for GM screening[11]. For reliable and precise PCR assays, the target sequences along with species-specific endogenous reference genes such as the *UGPase* gene[12] and *ST-LS1* gene[13] for potato may also be detected.

In the present study, two sets of designed primer pairs specific to the sequence of *AmA1* gene were employed for detection of GM potato with improved protein quality. Qualitative multiplex PCR assays were also developed for quick and simultaneous detection of *AmA1* gene, *CaMV* 35S promoter, *nos* terminator, *nptII* marker gene and endogenous *UGPase* gene in duplex, triplex and quadruplex formats. The established limits of detection for the simplex assays for *AmA1* gene were as low as 0.01%.

Plantlets of GM potato (*Solanum tuberosum* L.) line AmA KBD-5 with *AmA1* gene along with the non-GM line were provided by Central Potato Research Institute (CPRI), Shimla, developed in collaboration with National Institute of Plant Genome Research (NIPGR), New Delhi. Genomic DNA was extracted from the young leaves of two-week-old plantlets of GM and non-GM potato using DNeasy Plant Mini Kit (Qiagen, Hildon, Germany) according to the manufacturer's instructions. The quantity and quality of isolated DNA were assessed using a UV spectrophotometer (DU 640 Spectrophotometer, Beckman, USA). The DNA samples were then diluted to a final concentration of 20 ng/μl and used as stock solution for GM analysis.

Two pairs of primers using the sequence GenBank accession no. AF491291 of 2551 bp *AmA1* gene from *A. hypochondriacus* and the primer pair for amplification of the 515 bp region of *nptII* gene were designed with Primer 3 software. The published sequences of primer pairs, i.e. p35S-cf-3/p35S-cr-5 amplifying 123 bp of *CaMV* 35S promoter[14], tNOS-2-5′/tNOS-2-3′ primer pair amplifying 151 bp of *nos* terminator[15] and UGP-af7/UGP-af8 amplifying 88 bp of *UGPase* gene[12] were used. The primers were synthesized by Bioserve, Biotechnologies (India) Pvt Ltd. The details of primers used in the study are listed in Table 1.

Simplex PCR for *AmA1* gene was performed in 20 μl reaction volume containing a total of 100 ng template DNA per reaction, 1x polymerase buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl), 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.5 U *Taq* DNA polymerase (MBI Fermentas) and 0.25 μM each of forward and reverse primers. Amplification reactions were performed on PTC-200 Programmable Thermal Cycler (MJ Research, MA) under the following programme: initial denaturation at 94°C for 10 min followed by 40 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min, primer extension at 72°C for 1 min, and final extension at 72°C for another 8 min.

Multiplex PCR assays were performed using a primer concentration of 0.4 μM for *AmA1*, 0.1 μM for *CaMV* 35S promoter, 0.25 μM for *nos* terminator, 0.5 μM for *nptII* and 0.15 μM for *UGPase*. In multiplex PCR, the temperature profiles and other PCR conditions specific for amplification of the *AmA1* gene were used. Multiplex PCR was performed in duplex, triplex and quadruplex formats as follows:

(i) Duplex PCR: simultaneous detection of *AmA1* with 35S promoter; *AmA1* with *nos* terminator; *AmA1* with endogenous *UGPase* gene.

(ii) Triplex PCR: simultaneous detection of *AmA1*, *nptII* marker gene and *UGPase* gene.

(iii) Quadruplex PCR: simultaneous detection of *AmA1*, 35S promoter, *nos* terminator and *UGPase* gene in a single reaction.

The PCR-amplified products of simplex PCR were resolved on 2.0% (w/v) agarose gel (Lonza, Rockland, ME, USA) stained with ethidium bromide using 1X TBE as running buffer on horizontal electrophoresis, visualized under UV light and photographed using Gel Documentation Imaging System (Alpha Innotech, USA). For multiplex PCR, 4.0% (w/v) metaphor® agarose gel (Cambrex Bio Science Rockland, Inc. Rockland, ME, USA) was used.

Using simplex PCR, amplicons of the desired size of 171 and 167 bp of *AmA1* gene were detected using two primer pairs AmA–171-F/R and AmA–167-F/R, respectively. For sensitivity experiments,

Table 1. Primers used in the present study

Gene	Primer	Sequence (5′–3′)	Expected product size (bp)	Source
AmA1	AmA–171-F	CAAAGGTGGCTCATCAAATG	171	Present study
	AmA–171-R	AATCATGCACATCCGACCTA		
	AmA–167-F	CAAAGGTGGCTCATCAAATG	167	Present study
	AmA–167-R	ATGCACATCCGACCTAAACA		
nptII	nptIIF	GGGCGCCCGGTTCTTTTTG	515	Present study
	nptIIR	ACACCCAGCCGGCCACAGTCG		
CaMV 35S promoter	p35S-cf-3	CCACGTCTCAAAGCAAGTGG	123	Lipp *et al.*[14]
	p35S-cr-5	TCTCTCAAATGAAATGAACTC		
nos terminator	tNOS-2-5′	GTCTTGCGATGATTATCATATAATTTCTG	151	Lee *et al.*[15]
	tNOS-2-3′	CGCTATATTTTGTTTTCTATCGCGT		
UGPase	UGP-af7	GGACATGTGAAGAGACGGAGC	88	European Commission Protocol[12]
	UGP-af8	CCTACCTCTACCCCTCCG		

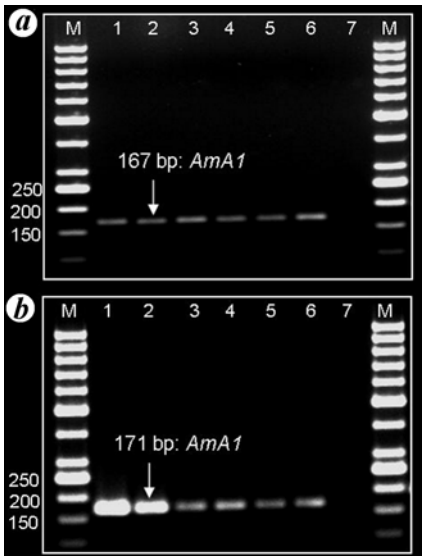

Figure 1. Sensitivity of the PCR assay using designed primers for *AmA1* gene. ***a***, Primer pair AmA–167-F/R. ***b***, Primer pair AmA–171-F/R. Lane M, 50 bp DNA ladder; lanes 1–7, Amplification of serial dilutions of GM potato with *AmA1* gene with 100, 10, 1.0, 0.1, 0.05, 0.01 and 0% respectively.

Table 2. Calculation of 'genetically modified organism' (GMO) genome copies in GM potato with *AmA1* gene as revealed by limit of detection experiment

Sample no.	GM DNA	Non-GM DNA	GM%	GMO genome copies
1	100	0	100	5.5×10^4
2	10	90	10	5.5×10^3
3	1	99	1	5.5×10^2
4	0.1	99.9	0.1	55
5	0.05	100	0.05	27.5
6	0.01	100	0.01	5.5
7	0	100	0	0

Source: Arumuganathan and Earle[16].

the limits of detection for simplex assays were assessed using the serial dilutions of 20 ng/µl DNA sample of GM potato (100% GM) with non-GM DNA with different percentages of GM trait, i.e. 100, 10, 1.0, 0.1, 0.05 and 0.01% (Table 2). A volume of 5 µl of the serially diluted DNA was used for PCR. Both sets of the designed primers, i.e. AmA–171-F/R and AmA–167-F/R amplified 171 and 167 bp of *AmA1* gene, respectively, in all the serial dilutions of GM potato, whereas no amplicon was detected in non-GM potato (Figure 1). The copy number of 'GMO' genome for each serially diluted reference DNA sample was calculated on the basis of the nuclear DNA content[16]. Though the standardized detection assay is qualitative, it helps in the identification of GM potato with a detection limit as low as 0.01%. The potato nuclear DNA content has been reported as 3.58 pg per two copies (2C) and accordingly, the number of copies of GM trait in 100 ng of potato DNA will be approximately 55,000. The reported data showed that GM DNA with 0.01% GM content representing 5.5 copies for improved protein quality in potato can easily be detected. The reported sensitivity meets the Supreme Court of India's stipulation for development of a protocol for testing contamination up to 0.01% prior to conducting field trials of GM crops[6].

Using multiplex PCR, several target DNA sequences can be screened for and detected in a single reaction with more precision. The duplex, triplex and quadruplex assays were performed using the AmA–171-F/R primer pair along with different combinations of primers of *nptII* gene, *CaMV* 35S promoter, *nos* terminator and endogenous *UGPase* gene; and only specific amplicons of the expected size were detected in GM potato. Using duplex PCR in three different combinations, an amplicon of 171 bp of *AmA1* gene was detected simultaneously with the amplicon of (a) 88 bp of endogenous *UGPase* gene, (b) 151 bp of *nos* terminator and (c) 123 bp of 35S promoter (Figures 2 *a–c*). In triplex PCR, three target sequences, i.e. 171 bp of *AmA1* gene, 515 bp of *nptII* marker gene and 88 bp of endogenous *UGPase* gene were simultaneously amplified in GM potato (Figure 2 *d*). Similarly, using quadruplex PCR, four targets, i.e. 171 bp of *AmA1*, 123 bp of 35S promoter, 151 bp of *nos* terminator along with 88 bp of endogenous *UGPase* gene were simultaneously amplified in GM potato (Figure 2 *e*). The desired amplicon of 88 bp for *UGPase* gene, being the endogenous gene of potato, was also amplified in non-GM potato. The potato specific reference gene was included as internal control to evaluate DNA quality and PCR efficacy, thus reducing the risk of false negatives.

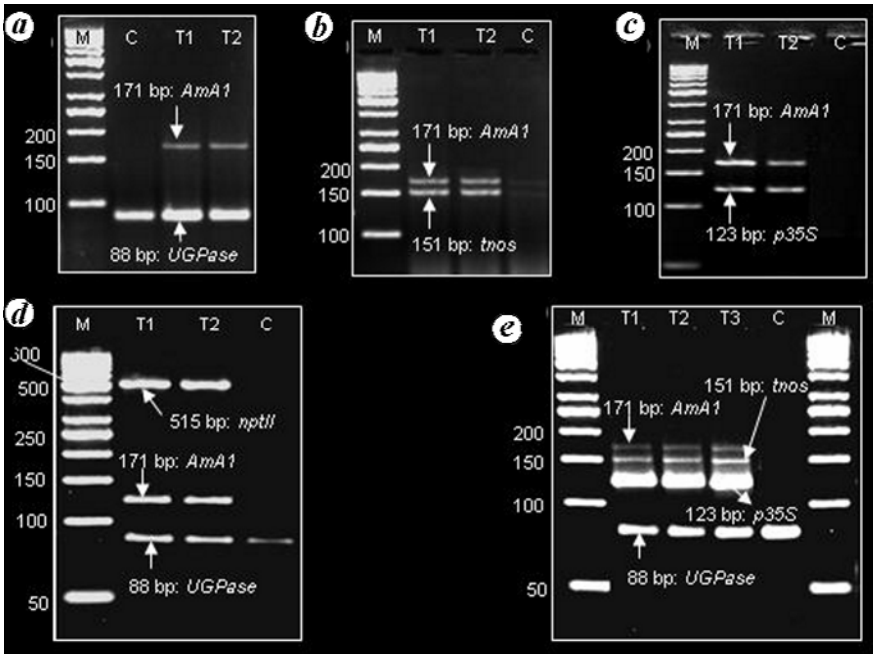

Figure 2. Multiplex PCR assays for detection of GM potato with *AmA1* gene. ***a*–*c***, Duplex PCR for simultaneous detection of *AmA1* gene along with endogenous *UGPase* gene (***a***), *nos* terminator (***b***), and *CaMV* 35S promoter (***c***). ***d***, Triplex PCR for simultaneous detection of *AmA1* gene, *nptII* marker gene and endogenous *UGPase*. ***e***, Quadruplex PCR for simultaneous detection of *AmA1* gene, *CaMV* 35S promoter, *nos* terminator and endogenous *UGPase* gene. Lane M, 50 bp DNA ladder; lanes T1–T3, GM potato with *AmA1* gene; lane C, Non-GM potato.

In the present study, sensitive, cost-effective, precise and efficient multiplex PCR assays in duplex, triplex and quadruplex formats have been developed for detection of *AmA1* gene, *CaMV* 35S promoter, *nos* terminator, *nptII* marker gene and endogenous *UGPase* gene in GM potato for better protein quality. The reported detection limit for simplex PCR assays was as low as 0.01%. Hence, the developed detection assays will be of immense use to meet the Supreme Court of India's stipulation for establishment of a protocol for testing contamination up to 0.01% prior to conducting field trials of GM crops and for compliance of regulatory obligations.

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