available for the livestock. Detailed spatial analysis indicated the majority of districts with less than 100 kg/ha methane flux. Few districts in Bihar, Haryana, Karnataka, Kerala, Punjab, Uttar Pradesh and West Bengal reported methane flux in the range 101–250 kg/ha. These outputs of methane emission inventory from the Indian livestock are important inputs for generating spatial integrated multi-source methane emission inventory at the national level.

5. MOEF, INCC-UNFCCC, India’s Initial National communication to the United Nations Framework Convention on Climate Change.

Molecular diagnosis of transgenic tomato with osmotin gene using multiplex polymerase chain reaction

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The requirement for upgradation of analytical methods for the detection of genetically modified (GM) crops is increasing at a fast pace, with a quantum jump in the area of GM crops being grown globally to meet the regulatory and international trade requirements. In the present study, standardization of multiplex polymerase chain reaction (MPCR) for the detection of

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GM tomato with osmotin gene was undertaken. In the MPCR, two pairs of designed primers specific for the inserted osmotin gene from Nicotiana tabacum in the transgenic tomato were used along with primers for 35S-promoter from Cauliflower Mosaic Virus and LAT 52 (endogenous late anther tomato) gene for simultaneous detection of the osmotin transgene, 35S promoter and endogenous LAT 52 gene. Internal control target (LAT 52 gene) was included both to assess the efficiency of all PCR reactions and to eliminate any false negatives. Molecular analysis using MPCR of the GM tomato revealed the detection of osmotin gene, CaMV 35S promoter and endogenous LAT 52 gene simultaneously in a single amplification reaction comprising 35 cycles with 59°C annealing temperature. MPCR detection results of GM and non-GM tomato suggest that the reported MPCR protocols would be suitable for detection and monitoring of transgenic tomato with osmotin gene.

Keywords: Genetically modified tomato, molecular diagnosis, multiplex PCR, osmotin gene.

TOMATO (Lycopersicon esculentum L.), belonging to the family Solanaceae, is an economically important crop in many countries, including India. Genetically modified (GM) tomato has been approved for commercialization in many countries since the first GM tomato ‘Flavr Savr’ was permitted for planting on large scale in 1994. In 2008, the global area of biotech crops continued to soar and showed an increase of 12% (10.7 m ha) between 2007 and 2008, reaching 125 m ha (ref. 1). With the development and commercialization of GM crops, the need for detection of GM organisms (GMOs) has become important for international trade of agricultural products. Concerns have been raised in the use of GM technology leading to potential health and environmental risks; one of these has been the proper labelling of food products produced using GM technology. As the genetic modifications using recombinant DNA techniques may cause biosafety issues related to the environment and human health, methods that can detect transgenes in GM plants need to be put in place. Polymerase chain reaction (PCR) has been widely used to confirm the presence of GMOs and to ensure the reliability of labelling systems.

High-yielding crop varieties with tolerance to abiotic stresses such as drought and salinity are always in demand for crop improvement programmes. Introduction of stress-tolerant genes from wild tomato species into tomato cultivars had initiated the breeding for resistance. Currently, modern molecular approaches are being utilized to develop cultivars that are tolerant to abiotic stresses. Substantial progress has been made in the identification of genes involved in abiotic stress tolerance, and their transfer to crops of economic importance for increased stress tolerance. Osmotin is a stress-responsive protein adapted to salinity and desiccation, and accumulates in salt-adapted cells. Overexpression of osmotin induces proline accumulation and confers tolerance to osmotic stress in transgenic tobacco. Singh et al. hypothesized that the synthesis of osmotin protein could induce synthesis and accumulation of certain solutes or could be involved in metabolic or structural changes.

Ouyang et al. transformed tomato (Lycopersicon esculentum cv. ‘A53’) with a tobacco osmotin gene and a bean chitinase gene using Agrobacterium to develop germplasm with improved Fusarium wilt resistance. Using PCR and Southern blot hybridization, stable integration of the transferred genes into the genome at various insertion sites was confirmed.

In India, successful integration of osmotin gene through Agrobacterium-mediated transformation for enhancing cold tolerance has been undertaken in tomato variety ‘Pusa Ruby’. In vitro selection against selectable marker kanamycin and PCR analysis of regenerated tomato plantlets for promoter (CaMV), npt-II (neomycin phosphotransferase II) and osmotin gene has confirmed the gene integration.

In 2006, Bansal and his group successfully developed transgenic tomatoes overexpressing osmotin gene, which were evaluated for drought and salt stress in the phytotron facility of the Indian Agricultural Research Institute (IARI), New Delhi, and the selected tolerant lines were tested in contained field trials.

PCR-based methods are the most common DNA detection methods for identifying the presence of GMOs. These methods are sensitive, and require small aliquots of material for GM analysis. PCR methods are not only used for the identification of GM products, but also for quantification purposes. To make the screening procedure robust, along with the detection of target sequences, plant species-specific reference gene should also be detected as an internal control. LAT 52 (Late Anther Tomato) gene is an anther-specific gene from tomato abundantly expressed during pollen maturation and is present in a single copy in the tomato genome. LAT 52 gene has been successfully used as a tomato endogenous reference gene in both the qualitative and quantitative detection of transgenic tomatoes, even for some processed foods derived from transgenic and non-transgenic tomatoes.

Multiplex PCR (MPCR) is a variant of the conventional PCR, which includes two or more pairs of primers in a single reaction to amplify corresponding genes simultaneously with the same sensitivity and specificity as PCR. MPCR has been used to detect the transgenes in different GM crops like soybean, maize, canola, squash and cotton. The advantage of multiplex methods is that fewer reactions are required to test a sample for the presence of GMO-derived DNA and it is more reliable and cost-effective.

In the present study, MPCR-based DNA assay for simultaneous detection of multiple target sequences, viz. osmotin
gene, CaMV 35S promoter sequence and endogenous LAT 52 gene in GM tomato has been developed. Internal control target (LAT 52 gene) was included both to assess the efficiency of all reactions and to eliminate any false negatives.

The seeds of two lines of transgenic tomato (line 528 and line 564) with osmotin gene along with the non-transformed tomato seeds were procured from National Research Centre on Plant Biotechnology (NRCBP), New Delhi, and were grown in the National Containment Facility at National Bureau of Plant Genetic Resources (NBPR), New Delhi.

Genomic DNA was extracted and purified from fresh leaves of 5–6-week-old seedlings of transgenic as well as non-transgenic tomato lines using modified CTAB extraction method. To tackle the problem of phenolics, 0.2% poly vinyl pyrrolidone (MW 40,000) was used in the extraction buffer. The quality of DNA was evaluated from the 260/280 nm UV absorption ratios and by agarose gel electrophoresis. The isolated DNA was quantified using VersaFluor™ Fluorometer. DNA samples were diluted to a final concentration of 5 ng/μl.

Two pairs of primers for detection of osmotin gene were designed using the sequence (GenBank accession no. X95308) of 2033 bp Nicotiana tabacum (common tobacco) osmotin gene with Primer Select 5.05 software (DNASTAR Inc., USA; Table 1). For the detection of endogenous LAT 52 gene, the primer pair Lat1/ Lat2 amplifying 92 bp band and for 35S promoter, the primer pair 35S-F/R amplifying 195 bp were used. The primers were synthesized by Operon Technologies, Inc., USA and Bioserve Biotechnologies (India) Pvt Ltd, India.

The protocols for individual as well as for MPCR were standardized using different PCR components and temperature regimes for each primer pair to detect osmotin gene, 35S promoter sequence and endogenous LAT 52 gene in the transgenic tomato lines. PCR was performed in 20 μl reaction mixture containing 25 ng template DNA, 1X polymerase buffer, 1.5 mM MgCl2, 200 μM dNTPs, 0.5 U Taq DNA polymerase and 0.2 μM each of forward and reverse primers. Gradient PCR was performed to select the annealing temperatures at which the primers give best results. Annealing temperatures ranging from 50 to 63°C and 52 to 60°C were used for osmotin 1 and osmotin 2 primer sets. Amplification reactions for osmotin gene using PCR was performed in PTC-200 Programmable Thermal Cycler (BioRad) under the following programme: initial denaturation at 94°C for 5 min followed by denaturation at 94°C for 30 s, primer annealing at optimum annealing temperature 59°C for 1 min, primer extension at 72°C for 1 min (in total, 35 cycles of the above programme was performed), and final extension at 72°C for another 8 min. MPCR was also performed under similar PCR conditions and temperature profiles using three sets of primers, viz. osmotin 1/osmotin 2, 35S and Lat. The PCR amplified products were resolved on 2% agarose gels stained with ethidium bromide using 1× TBE as the running buffer on horizontal electrophoresis, visualized under UV light and photographed using Gel Documentation System (Alpha Innotech, USA).

The two sets of primers for detection of the inserted osmotin gene were designed from the 2033 bp sequence (GenBank accession no. X95308) of osmotin gene in N. tabacum using Primer Select software. Gradient PCR (annealing temperatures ranging from 50 to 63°C and 52 to 60°C for osmotin 1 and osmotin 2 primer sets respectively) was performed to select the reliable annealing temperature. The most efficient primer annealing temperature for PCR reactions was 59°C for osmotin 1 and osmotin 2 primers. A 35-cycle protocol with 59°C annealing temperature using osmotin gene-specific primers was used. The results showed that the primers osmotin 1 and osmotin 2 amplified 418 and 353 bp products respectively, in the transgenic tomato lines 528 and 564, but no specific products were observed in the negative control sample (Figure 1 a and b). LAT 52 gene was used as a tomato endogenous reference gene in the molecular detection of transgenic tomatoes and an amplion of 92 bp was generated in both the transgenic lines as well as in non-transformed lines (Figure 2).

MPCR-based DNA assay for simultaneously detecting multiple target sequences in GM tomato was developed. MPCR was standardized to specifically amplify the osmotin gene, 35S promoter and LAT 52 gene, an endogenous tomato gene in a single assay. A 35-cycle protocol with 59°C annealing temperature using osmotin gene, 35S promoter and endogenous LAT 52 gene specific primers was used for simultaneous detection of these sequences in the transgenic tomato. In the first set of MPCR, the primers osmotin 1, 35S and Lat amplified specific products of 418, 195 and 92 bp respectively (Figure 3 a) in GM tomato. In another set, the primers osmotin 2, 35S and Lat amplified specific products of 353, 195 and 92 bp respectively, in GM tomato (Figure 3 b). No amplification products for osmotin gene and 35S promoter were detected in non-GM tomato, whereas 92 bp for endogenous LAT 52 gene was amplified in non-GM tomato line.

The GM crops have been approved for commercialization in many countries and some are in the pipeline of commercialization that have been approved for limited-scale field trials or multilocational field trials in India. To meet the legislative and regulatory requirements, the development of reliable and sensitive assays for GM detection is necessary. PCR is the most widely used analytical method for both qualitative and quantitative analyses of GM crops. Several research groups have developed a number of multiplex assays for GM detection, such as multiplexing for detection of five GM-maize events, viz. Bt11, Bt176, Mon810, T25 and GA21; detection of multiple target sequences in GM soybean (Roundup Ready™), detection of transgenes in GM


Table 1. Primers used for PCR amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'→3')</th>
<th>Gene</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmotin-1-F</td>
<td>ACTATGCTTCGCCGCTATT</td>
<td>Osmotin</td>
<td>418</td>
</tr>
<tr>
<td>Osmotin-1-R</td>
<td>ACCAGGGCCATTCACTATT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osmotin-2-F</td>
<td>CGGTTGATGGTGTTGAG</td>
<td>Osmotin</td>
<td>353</td>
</tr>
<tr>
<td>Osmotin-2-R</td>
<td>ACTATAGCCGTCAGGACATCTTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>35S-F</td>
<td>GTCCTCAAAATGCCATCA</td>
<td>CaMV 35S</td>
<td>195</td>
</tr>
<tr>
<td>35S-R</td>
<td>GATAGTGGATGTCGGCTCAGA</td>
<td>promoter</td>
<td></td>
</tr>
<tr>
<td>Lat 1</td>
<td>AGACACCGAGACAGTATTTG</td>
<td>Endogenous</td>
<td></td>
</tr>
<tr>
<td>Lat 2</td>
<td>TCTTGCTTCTTCTATCCAGAC</td>
<td>LAT 52</td>
<td>gene</td>
</tr>
</tbody>
</table>

Figure 1. Gradient PCR for detection of osmotin gene with primers (a) osmotin 1 amplifying 418 bp fragment and (b) osmotin 2 amplifying 353 bp fragment in transgenic tomato.

Figure 2. PCR for detection of endogenous LAT 52 gene with Lat1/ Lat2 primer amplifying 92 bp product in transgenic tomato. Lane M, 50 bp ladder; lane 1, Transgenic tomato line 528; lane 2, Transgenic tomato line 564; and lanes 3, 4, Non-transformed tomato lines.

Review Committee on Genetic Manipulation, Government of India. In view of this, the present study was undertaken to develop MPCR-based protocol for simultaneous detection of osmotin gene, 35S promoter gene and endogenous LAT 52 gene in GM tomato. Gradient PCR assays using two pairs of designed primers, osmotin 1 and osmotin 2, were performed to ascertain the optimum annealing temperature. The desired region of osmotin gene was amplified using the selected temperature range; however, 59°C was used as the optimum annealing temperature for better amplification in MPCR assays. For the detection of specific transgene osmotin, both the primers amplified the products of expected sizes and were further used along with primer pairs 35S-F, R and Lat 1, Lat 2 (ref. 21) in MPCR in triplex reactions. A 35-cycle protocol with 59°C annealing temperature was used for simultaneous detection of three sequences in the transgenic tomato. The primer sets for osmotin were found equally effective in MPCR assays as in individual PCR. The primer for tomato species-specific endogenous LAT 52
gene was incorporated in the MPCR assay to evaluate the PCR efficacy, thus reducing the risk of false negatives. Amplification of the desired products of 418 and 353 bp for osmotin gene and 195 bp for 35S promoter were detected only in GM tomato lines, whereas LAT 52 gene was amplified in both GM as well as non-GM lines as it is the endogenous reference gene for tomato.

The method reported in this study can considerably reduce the time and cost of detection of GM tomato with osmotin gene, especially for the screening of a large number of samples.

In the present study, to detect the osmotin gene, 35S promoter sequence and endogenous LAT 52 gene in transgenic tomato, a robust MPCR protocol has been developed, which is highly sensitive and efficient. More than one target sequence can be detected in a single assay under the same reaction conditions. The reported detection method will be of immense use to meet the regulatory obligations and legal requirements.

Production of andrographolate from adventitious root cultures of Andrographis paniculata

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Adventitious roots were induced directly from leaf segments of Andrographis paniculata on Murashige and Skoog (MS) medium with 5.3 μM α-naphthaleneacetic acid (NAA) and 30 g/l sucrose. Adventitious roots cultured in flasks using MS liquid medium with 2.7 μM NAA and 30 g/l sucrose showed higher accumulation of biomass (fresh and dry weight) and andrographolate within four weeks. Seven-fold increment of fresh biomass was evident in suspension cultures along with 3.5-fold higher andrographolate compared to natural plants. These results showed a great potentiality of adventitious root cultures for the production of andrographolate.

Keywords: Adventitious roots, Andrographis paniculata, Andrographolate, suspension cultures.

ANDROGRAPHIS PANICULATA Nees (Acanthaceae), commonly known as ‘Kalmegh’, has been widely used in India, Thailand, China and Malaysia for the treatment of hepatitis.¹² The plant is reported to possess protective activity against various liver disorders. The primary medicinal constituents of A. paniculata are andrographolate and related compounds which are diterpenoids showing antipyretic, antimalarial, anti-inflammatory, immunostimulatory and anticancerous activities.¹³-⁵

Plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites.⁶ Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to fast proliferation of cells/organisms and to a condensed biosynthetic cycle.⁷ Further, plant cell/organ cultures are not limited by environmental, ecological and climatic conditions and cells/organisms can thus proliferate at higher growth rates than the whole plant in cultivation.⁸ Callus cultures of A. paniculata have been reported⁹. However, no andrographolides were detected in the cultures. In the present study, induction and culture of adventitious roots of A. paniculata were conducted and production of andrographolate in adventitious root cultures was investigated.

Young leaves were collected from field-grown plants of A. paniculata Nees and were washed thoroughly in

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