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ACKNOWLEDGEMENTS. We thank the Director, IITM, Pune, for encouragement. This work was supported by the Department of Science and Technology, New Delhi.

Received 6 June 2008; revised accepted 21 October 2008

Molecular characterization of *Bt* cauliflower with multiplex PCR and validation of endogenous reference gene in Brassicaceae family

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***Bt* cauliflower is one of the important genetically modified (GM) crops approved for Biosafety Research Level 1 field trials in India. Towards developing reliable qualitative and quantitative PCR methods for detecting and monitoring GM crops, in the present study, molecular detection of *cryIAC* gene *CaMV 35S* promoter and an endogenous gene in *Bt* cauliflower was carried out by simplex and multiplex PCR. Furthermore, validation of S-locus receptor kinase (*SRK*) as the endogenous reference gene for the Brassicaceae family using simplex PCR has also been undertaken.**

Keywords: *Brassica oleracea* var. *botrytis*, endogenous reference gene, multiplex PCR, S-locus receptor kinase gene.

THE global area under cultivation of genetically modified (GM) crops has increased dramatically from 1.7 m ha in

1996 to 114.3 m ha in 2007, with 23 countries growing GM crops, including 12 developing countries and 11 industrial countries¹. Since more than two dozen GM crops are being developed and are under different stages of field-testing in India², concerns have been expressed about the potential risks associated with their impact to the environment, biological diversity and human health. To address these concerns, effective and reliable GM detection methods need to be put in place on priority to meet the national regulatory/legal and labelling requirements.

Multiplex PCR (MPCR) is a variant of conventional PCR, including two or more pairs of primers in a single reaction to simultaneously amplify corresponding genes. The MPCR-based detection method is most reliable, efficient and cost-effective and has also been successfully employed in various GM crops. A reliable MPCR protocol has been established for efficient detection of transgene neomycin phosphotransferase and endogenous gene 1-aminocyclopropane-1-carboxylate synthase in GM tobacco and tomato³. Molecular screening based on MPCR involving amplification of endogenous reference genes for soya and maize, *35S* promoter and *nos* terminator for the detection of GM soya and maize has also been developed⁴. Recently, the simultaneous detection of *vip3A*-type insecticidal gene, *nptII*, *35S* promoter and *nos* terminator in transgenic maize and cotton lines (Cot102) has been reported by MPCR assay⁵. The simplex and MPCR systems were standardized and employed to identify events Mon 1445 and Mon 531 from other GM cottons and GM crops⁶. Multiplex PCR assay to detect GM Roundup Ready soybean in foods has also been developed⁷. Two separate MPCR assays were developed⁸ for the detection of transgenic papaya line 55-1 and transgenic squash line CZW-3. In GM detection, for reliable MPCR, endogenous reference gene is an important component; and immense efforts have been made to validate the reference genes for different crops. Some of the validated endogenous reference genes are *zein* or *invertase1* genes for maize, *lectin* or *hsp* (heat shock protein) genes for soybean, *BnACCg8*, *cruciferin* or *HMGI/Y* genes for rapeseed, *LAT52* gene for tomato, *SPS* gene for rice and *Sad1* gene for cotton⁹. So far, validation of an endogenous reference gene for cauliflower has not been reported.

Brassica oleracea var. *botrytis* (cauliflower) is an important vegetable crop grown for its edible inflorescence. It is highly vulnerable to insect-pests that cause about 20–30% yield loss¹⁰. *Bt* cauliflower with *cryIAC* gene, developed by Sungro Seeds Research Ltd, India, has been approved for Biosafety Research Level 1 field trials.

In the present study, S-locus receptor kinase (*SRK*) gene, which is a female determinant of the genetic mechanism named as self-incompatibility (SI) in angiosperms, has been validated as an endogenous reference gene for the family Brassicaceae. The qualitative assay for detection of transgenes, viz. *cryIAC* and *CaMV 35S*

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Table 1. Primer pairs employed for PCR assay

NCBI accession no.	Primer	Sequence (5'-3')	Trait	Product length (bp)	Reference
V00141	35S-1/2	F-5'gctctacaatgccatca3' R-5'gatagtgggattgtgcgca3'	<i>CaMV</i> 35S promoter	195	14
X79432	SRK	F-5'ttggaggctattcgacacagagg3' R-5'caagccaatccatgaagcagtc3'	Female determinant of SI-response	311	Present study
	Cry1Ac1	F-5'attcagegggcccaggttta3' R-5'cagcgatggcaagttagaagg3'	Insect resistance	219	Present study
	Cry1Ac2	F-5'cgcaaccctaccgtatcc3' R-5'ccatgatccacgccgcagacaa3'	Insect resistance	354	Present study



Figure 1. Simplex PCR for detection of *CaMV* 35S promoter, endogenous *SRK* gene and *cry1Ac* gene (with both primers cry1Ac1 and cry1Ac2) in *Bt* cauliflower and MPCR with three sets of primers, including SRK, 35S and cry1Ac1. Lane M, 100 bp ladder; lanes 1–3, 35 S promoter-specific primer; lanes 4–6, *cry1Ac* gene-specific primer (cry1Ac1); lanes 7–9, *cry1Ac* gene-specific primer (cry1Ac2); lanes 10–12, *SRK* specific primer; lanes 13–17: MPCR; lanes 1–5, 7–11, 13–16, *Bt* cauliflower; lanes 6, 12, 17, Non-transgenic cauliflower.

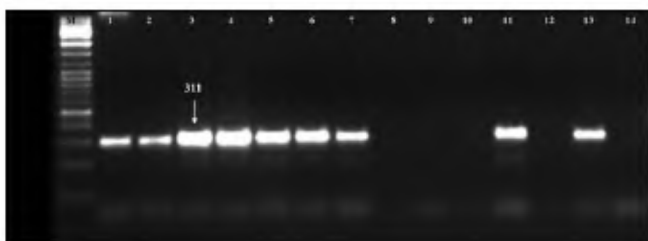


Figure 2. Simplex PCR for testing primer for *SRK* gene with crops of family Brassicaceae and other crops. Lane M, 100 bp ladder; lanes 1, 2, Cabbage (*Brassica oleracea* var. *capitata*); lanes 3, 4, Non-transgenic cauliflower (*B. oleracea* var. *botrytis*); lanes 5, 6, *Bt* cauliflower (*B. oleracea* var. *botrytis*); lane 7, Broccoli (*B. oleracea* var. *italica*); lanes 8, 9, Potato (*Solanum tuberosum*); lane 10, Papaya (*Carica papaya*); lane 11, Turnip (*Brassica napus*); lane 12, Peanut (*Arachis hypogaea*); lane 13, Mustard (*Brassica juncea*); lane 14, Tomato (*Solanum esculentum*).

promoter, and endogenous reference gene in *Bt* cauliflower by simplex and MPCR assays are also reported.

For validation of *SRK* as an endogenous reference gene, ten crops, viz., cauliflower (*B. oleracea* var. *botrytis*), cabbage (*B. oleracea* var. *capitata*), broccoli (*B. oleracea* var. *italica*), mustard (*B. juncea*), turnip (*B. napus*), brinjal (*Solanum melongena*), potato (*S. tuberosum*), tomato (*Lycopersicon esculentum*), papaya (*Carica papaya*) and

peanut (*Arachis hypogaea*), in which transgenic research is being undertaken, have been selected. The leaf samples of *Bt* cauliflower and non-*Bt* cauliflower containing *cry1Ac* gene were procured from Sungro Seeds Research Ltd, India.

The genomic DNA was extracted and purified from fresh leaves of 4–5-week-old seedlings of non-transgenic brinjal and tomato grown in the National Containment Facility, National Bureau of Plant Genetic Resources, New Delhi, from seeds of broccoli, mustard, turnip and peanut, from peel of potato and papaya, and from leaf samples of *Bt* and non-*Bt* cauliflower by modified CTAB method¹¹. The quality of DNA was checked by agarose gel electrophoresis. The quantity of isolated DNA was measured by Versa Flour™ Fluorometer and then diluted to a final concentration of 5 ng/μl. Two primer pairs for the detection of *cry1Ac* gene¹² and one for *SRK* gene were designed with Primer Select 5.05 software (DNASTAR Inc., USA; Table 1). *SRK* gene sequence for primer designing was obtained from NCBI (National Center for Biotechnology Information) database, GenBank accession number: X79432. For the detection of *CaMV* 35S promoter, the primer pair 35S-1/2 was used¹³. Synthesized primers were provided by M/s Genetix Biotech Asia Pvt Ltd. Each reaction mixture contained 1× PCR buffer, 200 μM of each dNTP, 0.2 μM of each primer, 25 ng of each DNA sample and 1U *Taq* DNA polymerase (MBI Fermentas), and the final reaction volume was 20 μl. The amplification reaction was run in the PTC-200 programmable thermal cycler (MJ Research) according to the program: one cycle of initial denaturation at 95°C for 5 min, 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 58.6°C, extension for 1 min at 72°C and a final extension for 8 min at 72°C. The PCR amplification products were resolved on 2% agarose gel and visualized under UV light using Gel Documentation System (Alpha Innotech, USA).

Two sets of MPCRs: (i) 35S-1/2, SRK, cry1Ac1 (Figure 1) and (ii) 35S-1/2, SRK, cry1Ac2 were carried out with primers for *CaMV* 35S promoter, *SRK* gene and *cry1Ac* gene (using two pairs of primers; cry1Ac1 and cry1Ac2). In non-transgenic cauliflower, no amplicon was detected for *cry1Ac* gene as desired, but amplicon of 195 bp of

CaMV 35S promoter and 311 bp of *SRK* gene were detected. Whereas in transgenic cauliflower, all the selected genes were amplified with the expected product size of 195 bp for *CaMV 35S* promoter, 311 bp for *SRK* gene, 219 bp for *cry1Ac* gene using *cry1Ac1* primer pair and 394 bp for *cry1Ac* gene using *cry1Ac 2* primer pair. As the primer for *CaMV 35S* promoter had amplified the DNA sequence in non-transgenic cauliflower, which was not desired, therefore, PCR with *CaMV 35S* promoter-specific primer was repeated twice to confirm the results; the same results were obtained. One of the reasons may be that cauliflower is a host of *Cauliflower mosaic virus (CaMV)*, and thus due to the attack by *CaMV* on cauliflower crop, the *CaMV 35S* promoter sequence was amplified.

An ideal endogenous reference gene should not exhibit allelic variation among varieties of the same species, while it should present a consistently low copy number in the different cultivars. To investigate whether crops of the Brassicaceae family exhibit allelic variation within the amplified *SRK* sequence, simplex PCR of selected ten crops mentioned above, including five members of the Brassicaceae family was performed. The results showed that the designed primer for *SRK* gene had amplified the specific amplicon of 311 bp only in crops of the family Brassicaceae (cauliflower, broccoli, cabbage, mustard and turnip) and not in other crops, i.e. tomato, potato, brinjal, papaya and peanut, confirming its specificity to detect members of the Brassicaceae family only (Figure 2). The results indicate that there were no major sequence differences among the different crops of the family Brassicaceae in this amplified region. It has been demonstrated that the *SRK* gene is a suitable endogenous reference gene for the identification and quantification of *Bt* cauliflower and other GM crops of the family Brassicaceae. The developed MPCR assays offer a useful tool to detect and monitor *Bt* cauliflower with high efficacy and reliability to meet the regulatory requirements and solve legal disputes, if they arise.

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ACKNOWLEDGEMENTS. We are grateful to the Global Environment Facility-World Bank; and Ministry of Environment and Forests, Govt of India, for financial support in the form of Junior Research Fellowship to R.C. and Research Associateship to M.S. We acknowledge M/s Sungro Seeds Research Ltd, India for providing the *Bt* and non-*Bt* cauliflower leaf samples. We also thank the Director, National Bureau of Plant Genetic Resources, New Delhi, for providing the necessary facilities.

Received 30 April 2008; revised accepted 23 October 2008

Surface soil moisture changes during 2007 summer monsoon season derived from AMSR-E Land3 product

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Soil moisture is an important component of the hydrological cycle. It contributes significantly to the water and energy flux from the surface of the earth, which in turn drives the atmospheric circulation. In spite of its importance, information on soil moisture is difficult to get. In India, soil moisture-measuring stations are sparse considering its dynamic nature. Remote

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