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ORIGINAL RESEARCH PAPER

Detection and copy number estimation of the transgenic nucleotide sequences in an unknown GM event of *Oryza sativa*

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Abstract

The present study was designed to establish a qualitative detection method based on conventional and real time PCR assay to screen the commonly grown rice varieties for the presence of the *cry1Ac* gene. The detection of genetically modified rice in the screening process would necessitate accurate assay development and precise qualitative PCR tests complying with established procedures for the detection and characterization of transgenes in food grains. Such assay would not only enable the monitoring of transgene flow in local agricultural environment but also the characterization of different plant species produced with this transgene and its regulatory components. Thus, a reliable and quick screening assay was established for the qualitative detection of the transgene along with the promoter and selectable marker gene in genetically modified rice. By conventional PCR, a fragment of 215 bp was amplified with gene specific primers of *cry1Ac*. Primers for other transgenes such as *gna* and *bar* were also employed; however, no amplification was detected. The presence of the *p35s*, *sps*, and *nptII* genes was confirmed by qualitative real-time PCR. The specificity of the respective PCR products was checked through melt peak curve analysis. Sharp and precise melting temperatures indicated the presence of a single kind of PCR product in correspondence to each of the primers used. Moreover, the copy number of *cry1Ac* was estimated by $\Delta\Delta C_T$ method. It is proposed that the primer sets and experimental conditions used in this study will be sufficient to meet the requirements for molecular detection and characterization of the *cry1Ac* transgene and affiliated sequences in sorting out conventional rice varieties from the ones which are genetically modified. It will also help to monitor the ecological flow of these transgenes and other biosafety factors.

Keywords

GM rice; real time PCR; $\Delta\Delta C_T$ method

Introduction

Rice (*Oryza sativa* L.) is one of the major food crops of the world. Approximately, 2.7 billion people depend on rice as their major food source [1]. In 2007, the average annual consumption was about 197 kg per capita, whereas it provided 49 and 39% of the calories and proteins, respectively. In Pakistan, rice is the 2nd most important food crop after wheat. Globally, Pakistan ranks 14th and 6th in rice production and exports, respectively, which account for 6% of foreign exchange earnings. The rice crop is cultivated on 2.57 million hectares, mainly in Punjab and Sindh which account for about 88% of the total yield. There are many constraints to the production such as availability of suitable varieties and proper agronomic inputs due to which the average yield stands at ≈2240 kg/ha, which is very low as compared to the potential yield.

The spread and cultivation of unapproved rice varieties by the farming community is also a major problem because such varieties have not been tested for their resistance against biotic (mainly insects) and abiotic stresses, and suitable agronomic practices cannot be adopted according to the growth pattern and nutritional requirements of the crop at different growth stages. Admixing of the seeds of different varieties is also another issue which hampers a uniform production per given area of land.

Estimated data shows that the insect pests cause 5% of loss of the total output of rice annually [2]. Because of the complex mechanism of genetic resistance and the inherent difficulty and poor understanding of the genetic mechanism of resistance, the breeding of insect pest resistance in rice is difficult by conventional breeding methods. The insertion of exogenous insect resistance genes in rice and breeding of these rice varieties can effectively reduce the loss and stabilize rice yield [3]. With the rapid improvements in genetic engineering, transgenic rice with a number of genes encoding different proteins, such as *Bacillus thuringiensis* (*Bt*), protease inhibitors [4], and plant lectins [5], has been developed to improve resistance to insects. Among all these, the introduction of the *Bt* gene has emerged as the most effective protector [1]. The *Bt* gene encodes for a lethal toxin which is particularly toxic to the striped stem borer and yellow stem borer in the rice crop [6]. Field trials of *Bt* rice have indicated that genetically modified rice can bring multiple benefits, including better yields, a decrease in the use of chemical pesticides coupled with a reduction in the adverse effects of pesticides on farmer's health, an improved and enhanced quantity of the essential nutrients, and a reduced cost [7].

GM rice in Pakistan

The research and field trials of genetically modified rice in Pakistan began in the early 90s. The main aim then was to introduce resistance against bacterial pathogens in the elite rice cultivars. In 1997, a variety of basmati called 'Super Basmati' was transformed by the *Xa21* gene using a micro-projectile gun [8]. This GM rice was resistant to bacterial leaf blight. Thus, the crop plant, though it has been modified genetically long before (Tab. 1), its large scale trials and the transformation of other varieties has been on halt. It is because the rice is an export crop of Pakistan and there is a lack of a regulatory framework which could segregate non-GM and GM rice on a wide scale. Moreover, there has been a strict ban on GM rice grains in the export destination countries, particularly in the Middle East and the European Union [9].

Objective

Keeping in view the research status and the biosafety regulations in force, the present study aims to detect the presence of the transgene (*cry1Ac*) in locally grown rice varieties along with the quantification of the copy number of the exogenous gene in varieties which are found positive for a genetically engineered genome.

Tab. 1 Progress and status of GM rice development in various institutions of Pakistan.

Institute	Problem	Gene	Stage/level	Reference
CEMB*	Resistance to yellow stem borer and rice leaf folder	*cry1Ac*; *cry2A*	Field evaluation	[21]
		cry1Ac+cry2A	Lab and field evaluation	[22]
		cry1Ab	Lab and field evaluation	[23]
NARC**	Bacterial leaf blight	*Xa21*	Lab and green house study	[24]

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** National Agricultural Research Centre, Islamabad, Pakistan.

Material and methods

Samples of different rice varieties were collected from the grain market in Lahore. The grains were produced in the late season of 2012 in Punjab, Pakistan. All shipping and handling was conducted to protect the freshness and quality of the grains. On arrival at the laboratory, the grains were stored at 5°C before grinding and subsequent storage at 18°C until flour making. For each analytic sample, multiple DNA extractions/selected variety were made by using the Purelink Genomic DNA extraction kit (Invitrogen) according to the manufacturer's instructions. DNA concentration was calculated by using the spectrophotometer for an A_{260}/A_{280} nm ratio, which was ≈1.7. This result for concentration and purity was satisfactory to proceed with conventional and RT PCR. Prior to PCR, the extracted DNA was diluted in molecular biology grade water to give ≈500 ng DNA per 5 µL. For conventional and qualitative/quantitative real time PCR, primer pairs were selected according to the published resources with an authenticity to specific nucleotide sequences (Tab. 2). All the primers were synthesized by Vivantis Technologies (Malaysia).

Conventional PCR

PCR mixes per reaction included 0.5 uM of each primer and 5 µL of diluted DNA in a final volume of 50 µL of 1× PCR buffer (Platinum® PCR SuperMix, Invitrogen). The ICCC thermocycler was programmed for an initial denaturation at 95°C for 3 min followed by 40–50 cycles of denaturation for 30 s at 94°C, 30 s at 58°C (annealing), and for 30 s at 72°C (extension) with the final extension at 72°C for 10 min. The PCR products were analyzed on 2% agarose / ethidium bromide gel (Vivantis) along with 100-bp DNA mass ladder (Invitrogen), visualized by direct observation on a UV trans-illuminator, and the images were recorded using a gel documentation system (Bio-Rad, CA, United States).

Qualitative real time PCR (qRT PCR)

In order to carry out a sensitive detection of the transgenic nucleotides, qRT PCR was performed in an iCycler (Bio-Rad) with a final volume of 25 µL, which comprised

Tab. 2 List of the primers used for the identification of transgenic nucleotide sequences.

Gene	**Primer sequence**	**Bp size**	**Reference**
bar	F: ACCATCGTCAACCACTACATCG	430	[25]
	R: GCTGCCAGAAACCCACGTCAT		
gna	F: CGGATCCATGGCTAAGGCAAGTCTCCT	480	[26]
	R: CGGTACCTCATTACTTTGCCGTCACAA		
tnos	F: GAATCCTGTTGCCGGTCTTG	180	[1]
	R: TTATCCTAGTTTGCGCGCTA		
cry1Ac	F: GTTCGTTCTCGGACTAGTTG	228	[25]
	R: ACGGAGGCATAGTCAGCAGGACC		
sps	F: TTGCGCCTGAACGGATAT	81	[26]
	R: CGGTTGATCTTTTCGGGATG		
p35s	F: GCTCCTACAAATGCCATCA	195	[1]
	R: GATAGTGGGATTGTGCGTCA		

20 µL SybrGreen PCR Super Mix Universal (Invitrogen), 1 µL each primer, and 3 µL genomic DNA. The iCycler was programmed for an initial denaturation at 95°C for 3 min, followed by 50 cycles of denaturation for 40 s at 94°C, 30 s at 54°C (*tnos*, *p35s*) / 58°C (*bar*, *gna*, *cry1Ac*, *sps*; annealing), for 40 s at 72°C (extension) with the final extension at 72°C for 10 min. qRT PCR also included a melt curve peak analysis with temperatures ranging from 50°C to 95°C. qRT PCR was performed under similar conditions for the establishment of the standard curves and estimation of the *cry1Ac* copies. The reference endogenous gene was the sucrose phosphate synthase gene which is an established housekeeping gene in the rice genome.

Results

First of all, the varieties were sorted out in conventional PCR with the primers for *cry1Ac*, *p35s*, *tnos*, *gna*, and *bar* (Fig. 1, Fig. 2). As the plateau phase was not used for any calculation in the standard curve method and the important part of the curve was exponential, thus to save the run time (and also the reagents), the reaction was stopped at the peak of the exponential phase.

The varieties positive for the presence of *cry1Ac* were subjected to conventional PCR to determine the PCR product size on agarose gel (Fig. 3, Fig. 4), followed by qRT PCR for *p35s* (Fig. 5).

The selected varieties when tested for *p35s* gene presence by qRT PCR (SybrGreen Technology) resulted in sharp amplified curves for the target sequence. The PCR efficiency remained smooth during amplification and normal curves were obtained; moreover, each of the three repeats of a given variety showed an ample amplification of the *p35s* gene. The rice varieties for which the amplification curves for the *p35s* gene were obtained were subjected to conventional PCR, also so as to determine the PCR product size on agarose gel (Fig. 6).

Being a robust technique, qRT PCR confirms the presence of *cry1Ac* and *p35s* in certain varieties showing that the genome of these rice varieties has been modified (Tab. 3).

In this method, the standard curves of a reference gene (in this case the *sps* gene) and the target gene (*cry1Ac*) were established and used to calculate the ΔC_T value at a definite DNA dilution point. Efficiency of the standard curves was used to calculate the correct C_T values for the DNA concentrations used from the sample DNA.

For copy number estimation, 'Pakhraj' was the standard variety, i.e., the calibrator, because it was cultivated on a large area. Three DNA dilutions (100 ng, 200 ng, 400 ng) were run in triplet of each dilution point for amplification of *sps* (reference gene) and *cry1Ac* (target gene) alternatively. The average of the C_T values obtained for each of the dilution was drawn for the respective standard curve, each point representing the average values of three repeats (Fig. 7, Fig. 8).

The two curves show a reaction efficiency of 99.7% (*sps*) and 94.7% (*cry1Ac*). The R^2 values of the two curves were 0.997 and 0.947, respectively, which shows a highly efficient multiplication of the target genes. ΔC_T was calculated by subtracting the C_T value of *sps* from *cry1Ac* at a particular concentration in the standard curves (Tab. 4).

Three repeats of the sample variety 'Kaynat' were run for each of the *sps* gene and *cry1Ac* gene at approximately 100 ng of DNA concentration. The results were as below (Fig. 9).

The C_T values for the transgene *cry1Ac* were 42.62, 42.05, and 42.11, whereas for the reference gene *sps* 33.16, 33.22, and 34.05. The average C_T values for *cry1Ac* and *sps* were 42.26 and 33.48, respectively.

Based upon the respective standard curves, corrected C_T values for *cry1Ac* and *sps* were calculated according to the Eq. 1, which describes the exponential amplification of the PCR:

$$XT = X0(1 + EX)C_T X \qquad \textbf{Eq. 1}$$

where *X*T is the amount of target molecules (*cry1Ac* gene) at threshold cycles, *X*0 is the initial amount of the target molecule, *EX* is the efficiency of the amplification, and $C_T X$ is the threshold cycle of the target DNA amplification [16]. From the equation of

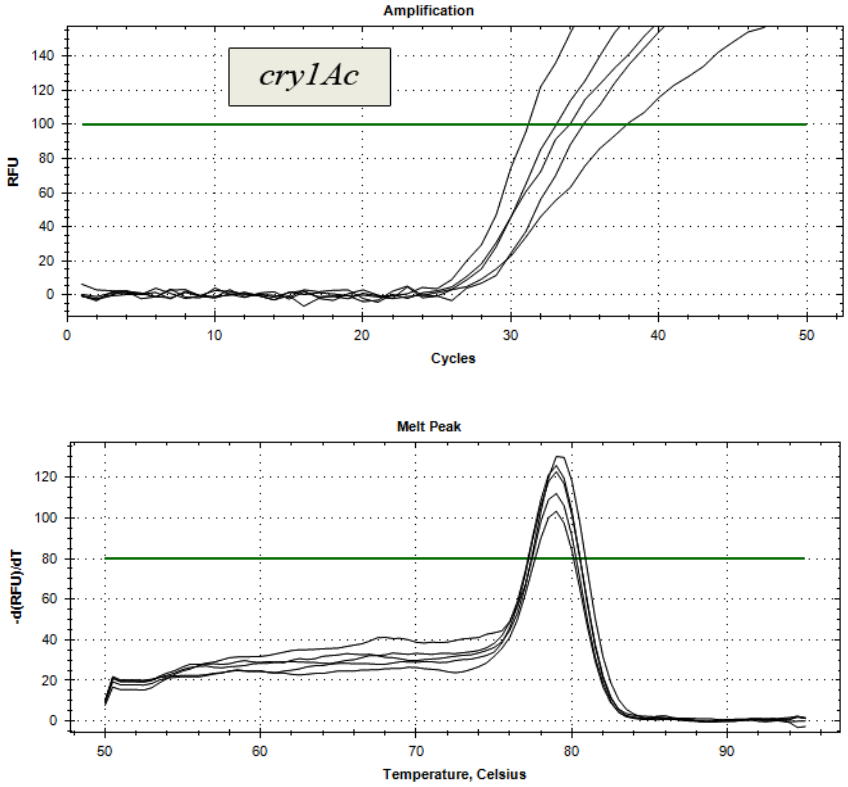

Fig. 1 Screening step: 17 varieties screened but only five showed amplification for the *cry1Ac* gene.

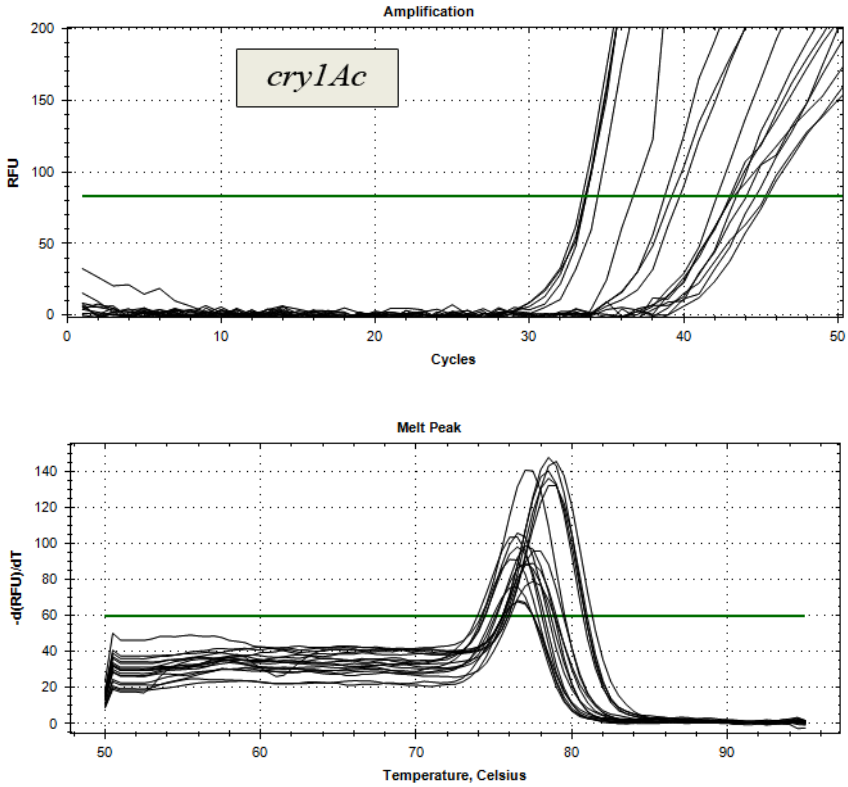

Fig. 2 The five varieties which showed amplification in the screening step were again run in triplets of samples/variety for qRT PCR with the *cry1Ac* gene plus one triplet of samples for the *sps* gene (the house keeping gene).

Fig. 3 The *cry1Ac* gene (215 bp) in rice varieties.

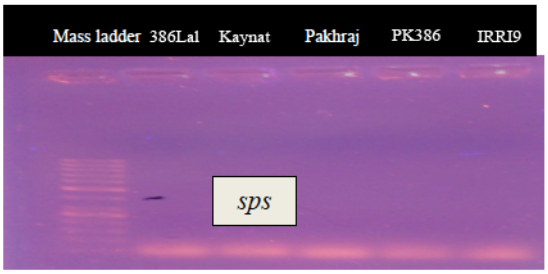

Fig. 4 The *sps* gene (81 bp) in rice varieties.

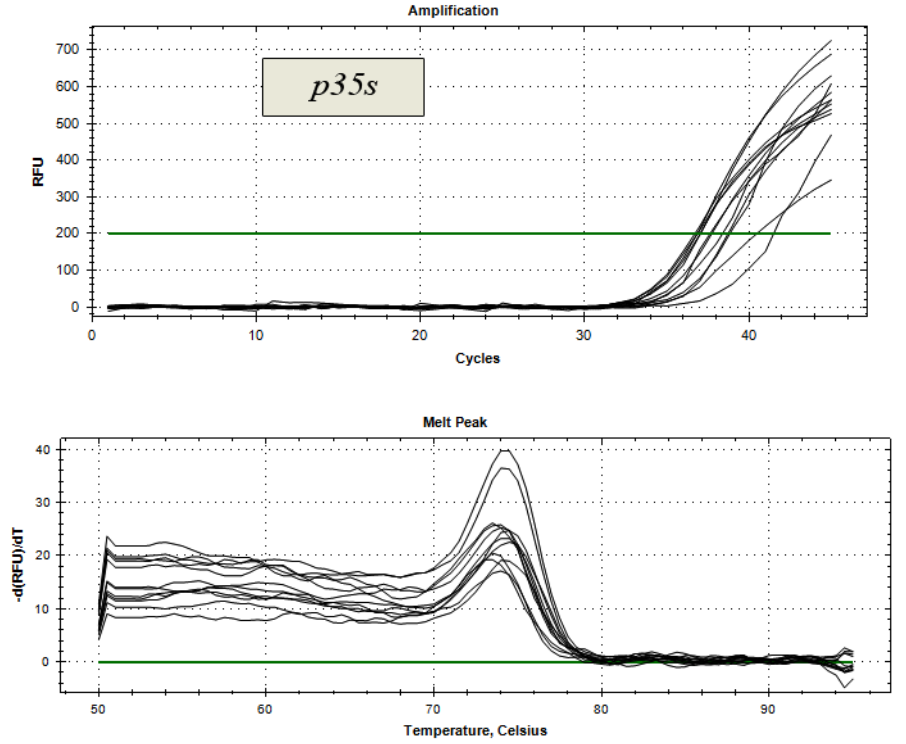

Fig. 5 The transgene *p35s* confirmed in five GM rice varieties each with two repeats plus two curves for *sps*.

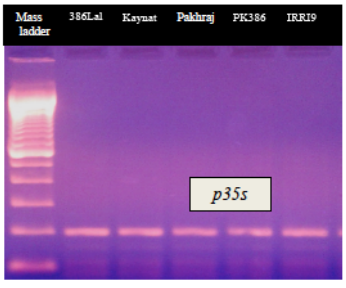

Fig. 6 The *p35s* (195 bp) in rice varieties.

Tab. 3 Detected transgenic nucleotides in rice varieties.

Serial No.	Variety	*p35s*	*cry1Ac*	*nos*	*gna*	Variety is GM/ conventional?
1	'386Lal'	+	+	–	–	GM
2	'Kaynat'	+	+	–	–	GM
3	'Pakhraj'	+	+	–	–	GM
4	'PK386'	+	+	–	–	GM
5	'IRRI9'	+	+	–	–	GM

Estimation of the transgene copy number by efficiency corrected $\Delta\Delta C_T$ method. "+" – present; "–" – absent.

E = 99.7% R^2 = 0.981 Slope = –3.330

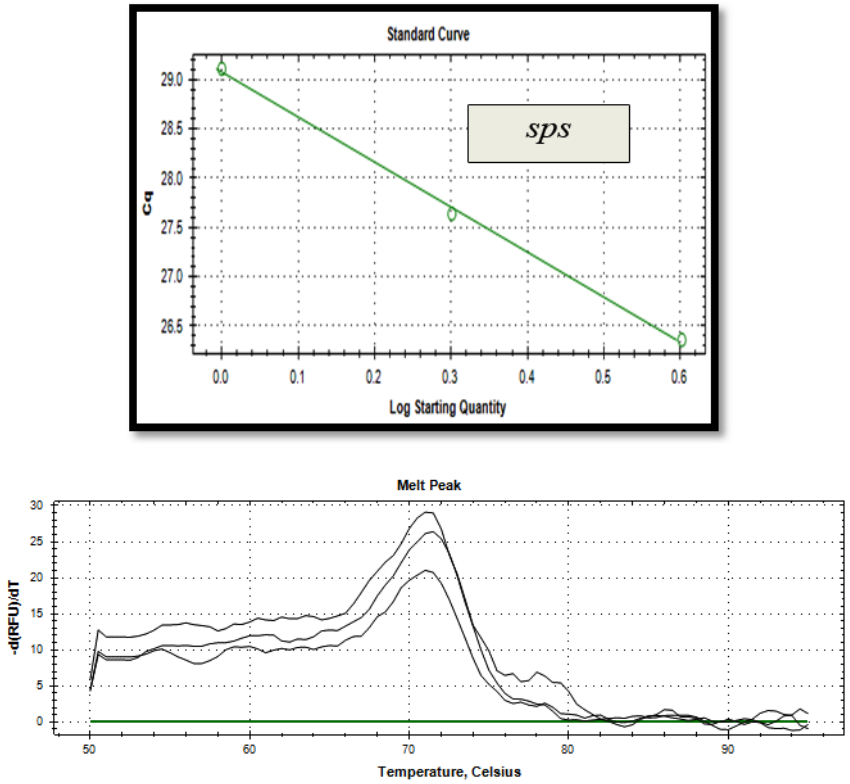

Fig. 7 Dilutions and standard curve for *sps*.

E = 94.7% R^2 = 0.934 Slope = –3.332

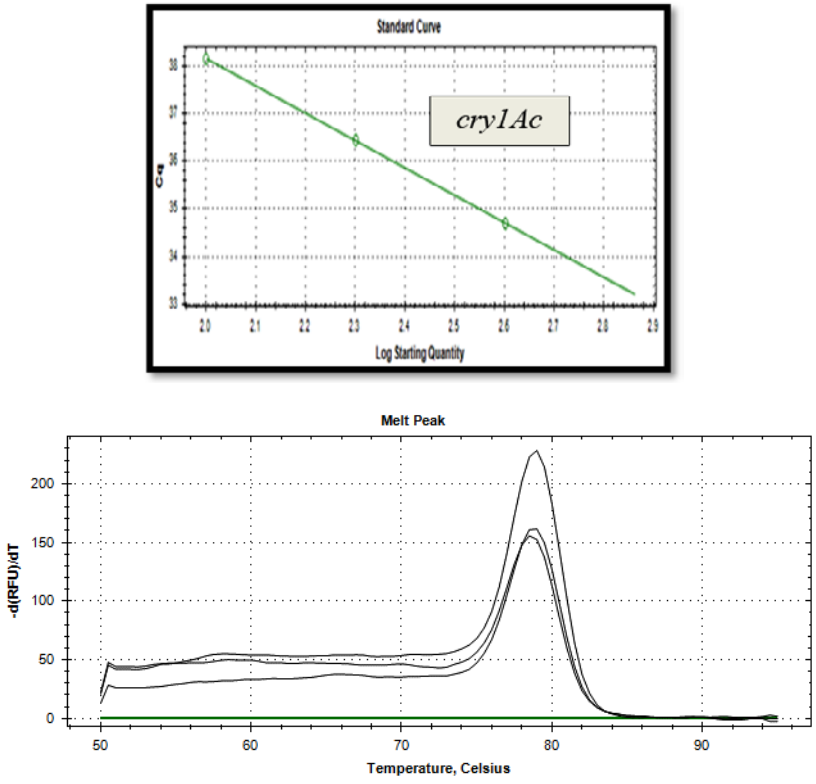

Fig. 8 Dilutions and standard curve for *cry1Ac*.

Tab. 4 Delta C_T values for *cry1Ac* and *sps* at specific DNA dilution points.

Serial No.	Dilution (ng)	*cry1Ac* C_T – *sps* C_T	Delta C_T
1	100	38.16 – 29.11	9.05
2	200	36.45 – 27.64	8.81
3	400	34.69 – 26.36	8.33

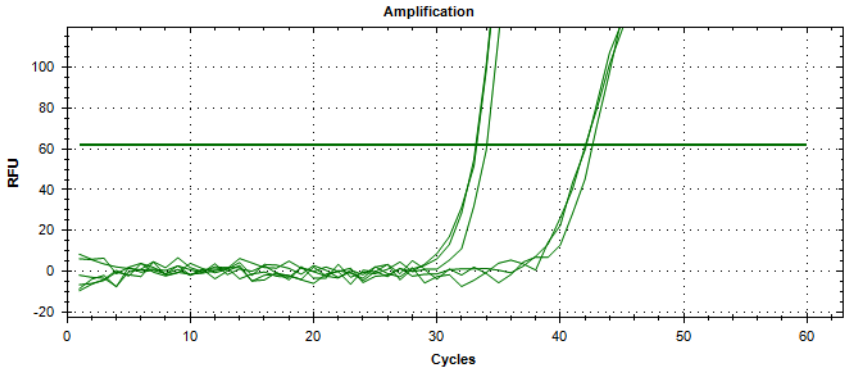

Fig. 9 Amplification of the *cry1Ac* and *sps* genes in the sample variety 'Kaynat'.

PCR amplification, the PCR efficiency (EX, ER) is obtained from the relative standard curve of the 'Kaynat' dilution for *cry1Ac* or *sps*.

Based upon the standard curve, corrected C_T values for the initial concentrations of *cry1Ac* were calculated as follows:

$X_{cry1Ac} = X0\ (1 + E_{cry1Ac})\ C_{T\ cry1Ac}$

$X_{cry1Ac} = 100\ (1 + 0.94)^{42.26}$

$X_{cry1Ac} = 145\,378\,809\,075\,550.82$

The C_T value according to the standard curve is:

$X_{cry1Ac} = X0\ (1 + E_{cry1Ac})\ C_{T\ cry1Ac}$

$145\,378\,809\,075\,550.82 = 100\ (1.94)\ C_{T\ cry1Ac}$

$1\,453\,788\,090\,755.50 = (1.94)\ C_{T\ cry1Ac}$

$\log 1\,453\,788\,090\,755.50 = C_{T\ cry1Ac} \log 1.94$

$C_{T\ cry1Ac} = \log 1\,453\,788\,090\,755.50 / \log 1.94 = 12.16/0.287 = 42.37$

Similarly, the corrected C_T value for the initial quantity of *sps* transcripts was also calculated based upon the respective standard curve:

$X_{sps} = X0\ (1 + E_{sps})\ C_{T\ sps}$

$X_{sps} = 100\ (1 + 0.99)^{33.48}$

$X_{sps} = 1\,012\,980\,297\,025.2525$

The C_T value according to the standard curve is:

$X_{sps} = X0\ (1 + E_{sps})\ C_{T\ sps}$

$1\,012\,980\,297\,025.25 = 100\ (1.99)\ C_{T\ sps}$

$10\,129\,802\,970.25 = (1.99)\ C_{T\ sps}$

$\log 10\,129\,802\,970.25 = C_{T\ sps} \log 1.99$

$C_{T\ sps} = \log 10\,129\,802\,970.25 / \log 1.99 = 10.00/0.299 = 33.44$

$\Delta C_T = 42.37 - 33.44 = 8.93$

Similarly, ΔC_T values for other varieties were calculated to find out the copy number of the *cry1Ac* integrations.

Validation of the procedure for estimation of the copy number

Due to the unavailability of the reference genotypes of GM rice in Pakistan (where so far no GM rice event has been commercialized for large scale production), the

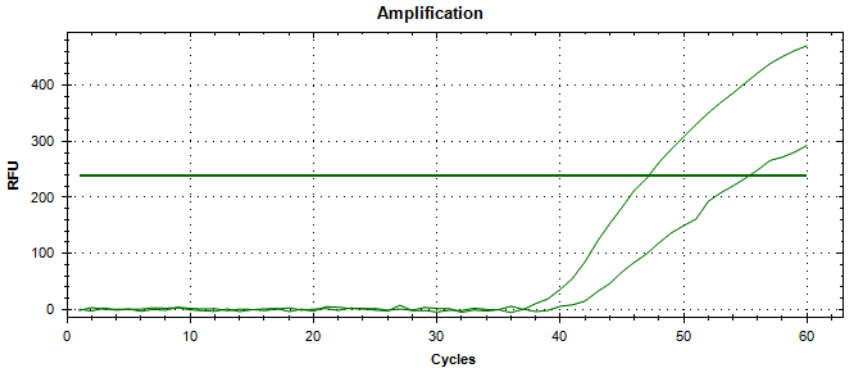

Fig. 10 Amplification of the *cry1Ac* and *sps* genes in the certified reference material of MON531 event.

Tab. 5 The *cry1Ac* integrations in the sample varieties with respect to 'Pakhraj' (calibrator variety).

Serial No.	Variety	ΔC_T calibrator	$-\Delta C_T$ sample variety	$\Delta\Delta C_T$	$2\Delta\Delta C_T$	Estimated gene copy number
1	'Kaynat'	9.05	8.93	0.12	1.086	1
2	'PK386'	9.05	9.25	−0.20	1.150	1
3	'Superfine'	9.05	9.44	−0.39	1.310	1
4	'IRRI9'	9.05	9.11	−0.06	1.040	1
5	'Pakhraj'	9.05	9.32	−0.27	1.200	1
6	CRM	9.05	8.90	0.15	1.100	1

certified reference material (CRM) of MON531 event (AOCS 0804-C by AOCS company) was used for validation of the procedure and calculations made in the assay. It must be noted here that MON531 is the only transgenic plant event commercially adopted in Pakistan. The event contains a single copy of the transgene *cry1Ac* (identified in the rice genome in this study) and is intended to be used as quality control or calibrant for the detection, identification, and/or quantification of GM events. The *sps* gene has already been validated as the reference gene in cotton, also [11–13].

Both of the *sps* and *cry1ac* genes were amplified in qPCR under identical conditions of PCR at a DNA concentration of 100 ng with the same primer sets as used in case of the rice varieties. Based upon the pre-determined standard curves, the resulting C_T values were used to calculate the copy number of the *cry1ac* gene (Fig. 10).

The C_T values for *cry1Ac* and *sps* were 55.49 and 46.76, respectively. Based upon the standard curve, corrected C_T values for *cry1Ac* and *sps* were determined by using Eq. 1: $C_{T\,cry1Ac} = 55.64$; $C_{T\,sps} = 46.74$; $\Delta C_T = 55.64 - 46.74 = 8.9$.

The ΔC_T value for the CRM was used to authenticate the assay (Tab. 5).

Discussion

The *cry1Ac* gene has been introduced into a multitude of crop plants for resistance against insect pests, particularly *Helicoverpa armigera* (Hubner). The genetically modified crops include cotton, maize, poplar, soybean, tomato, and rice. Reiting et al. [14] developed a cascade to distinguish and sort out different GM rice events based upon qualitative PCR for the CAMV-35S and NOS genes. Mahmood et al. [15] determined the copy number of *cry1Ac* and *cry2A* in locally developed transgenic lines by fluorescence in situ hybridization (FISH) technique only without a comparative method, e.g., qPCR. Currently, there is no assay available to determine the *cry1Ac*

copy number in transgenic rice by $\Delta\Delta C_T$ method. Thus, in the present study RT qPCR was coupled with the standard curve method to yield a robust assay to sort out the transgenic rice from the conventional non-GM varieties along with an estimation of the transgene copies. Instead of Southern blotting, MON531 CRM was used to validate the procedure and calculations in conditions identical to the GM rice varieties, because Southern blot can lead to an underestimation on several counts, including the insertion of more than one T-DNA copy at a single locus and the generation of DNA fragments very similar in size that are not resolved on agarose gels [16]. Moreover, studies involving the confirmation of the copy number by Southern blotting often lead to non-identical results as determined by qPCR method [17] because a single band on a Southern blot does not necessarily correspond to a single transgene copy [18]. The qPCR copy number assay also has an advantage over Southern blot analysis in the size of sample required for each assay where up to 1/20th concentration of DNA can be used in the assay.

The complete GMO detection analysis process consisted of the homogenization of the sample, purification of DNA, followed by the detection/identification of the transgene. The suitability of DNA extracts as templates for PCR-based transgene assays was checked independently in conventional and qRT PCR. The primers were selected from the published literature according to the event MON531 because the number of events of genetically modified organisms (GMOs) authorized in Pakistan has not increased since 2010 when a GM cotton event (MON531) was formally commercialized. The research and field trials of other GM plants have been carried out at different research institutes; however, none of these trials has led to the approval of a GM variety at the country level. In order to know the possible presence of transgenic nucleotides in rice varieties and to test the degree of compliance with the biosafety regulations in force, 17 commonly grown rice varieties were randomly selected for the screening phase, followed by confirmation of the transgene's presence in different steps (Fig. 11).

The presence of the transgenic sequence was considered positive when signals of amplification were observed before the 40th cycle. The result indicates the amplification of the target gene *cry1Ac* sequence in only five varieties, namely: '386Lal', 'Kaynat', 'Pakhraj', 'PK386 brown', and 'IRRI9', while all other varieties showed an absence of *cry1Ac*. Although the varieties were randomly selected from the local grain market, the presence of the *cry1Ac* gene (though in some of them) reveals the extent of the

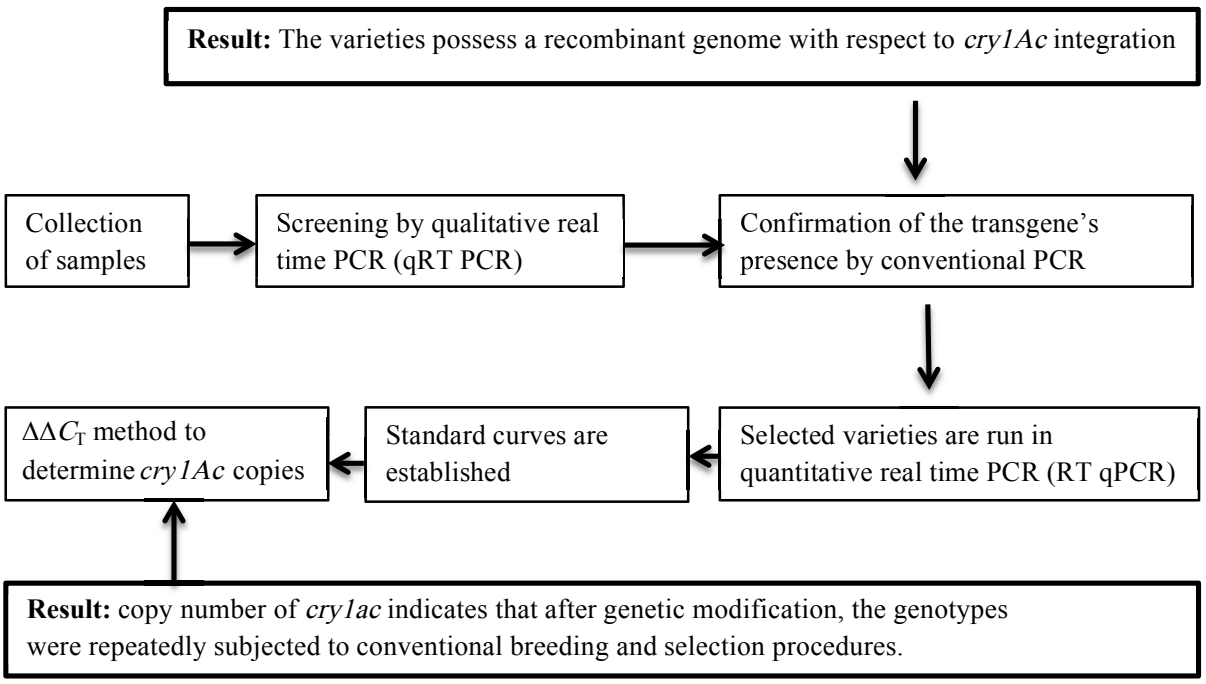

Fig. 11 The scheme of work performed.

spread of the transgenic grains in the country. The method of detection was PCR which is a highly robust technique to amplify specific segments of DNA occurring at a low frequency in a complex mixture of DNA sequences. In SybrGreen real time assays, the amplification plots and melt curves indicated the multiplication of the transgene with a confirmation that the product being formed was of a single kind, i.e., only the target gene sequence was amplified. In the real time runs, melt peak curves identify the particular product from primer dimers and other amplification artifacts. Thus, once it was established that the genome of the rice varieties was modified with the introduction of exogenous nucleotide sequences (*cry1Ac* and *p35s*), the number of *cry1Ac* integrations per copy of the genome was estimated. For this purpose, efficiency corrected $\Delta\Delta C_T$ method was used, i.e., the copy number of the target gene = $2^{\Delta\Delta C_T}$.

Concerns over the use of GMOs have led to myriad national regulations for transgenic plants in most countries. Labeling of GM food products has become an important part of the regulatory framework in many countries, including those in the European Union, the United Kingdom, Japan, Australia, New Zealand, and Thailand. The biosafety laws in Pakistan prohibit the spread of genetically modified rice at the commercial level. Thus, commercialization and cultivation of GM rice in Pakistan are restricted to laboratory and greenhouse trials. It is due to the fact the export destination of Pakistani Basmati rice are EU and Arab Gulf countries where there is a strong disapproval for the GM grains. In this connection, the rapid alert system for food and feed (RASFF) of the European Union issued five notifications related with the detection of GMO content in basmati rice imported from Pakistan (Tab. 6). As per summary record of the Standing Committee on the Food Chain and Animal Health held in Brussels on 12th and 13th March 2012 (section Genetically Modified Food and Feed and Environmental Risk), the response from Pakistan to a request from the Commission for information on GMO policy clearly confirmed that only *Bt* cotton was authorized for cultivation and that no trials, either confined or open field, had been authorized for any GM rice variety.

The transformation of the rice genome may be accomplished by different methods such as *Agrobacterium* mediated transformation, particle bombardment, etc., each with a varying efficiency of transformation and the copy number ranging from a single to multiple copy number. The inserted copies may be randomly inducted or placed at a single insertion site in the genome. The expression level and stability of the transgene copies though is positively influenced by a high number of the genome copies, multiple copies, however, may also lead to gene silencing [19]. In some other cases no relationship between the gene copy number and gene silencing has been found [20]. As no transformation method can control the number of transgene integrations, it is critical to identify the rice varieties with respect to transgene copy number to sort out the lines for further propagation. In this study, an estimation of the copy number reveals a single integration of the *cry1Ac*/genome copy, which is most probably the result of repeated selection and breeding of the GM varieties to sort out the genotypes with an optimal gene expression. The reference material with a known single copy of the transgene was used to validate the copy number estimation procedure. As per use of the assay developed, the transgene (*cry1Ac*) copy number in the certified reference

Tab. 6 RASFF notifications issued for GM rice content from Pakistan.

Serial No.	Reference	Notification from	Notification date	Product	Country of origin
1	2012.0388	Germany	14-03-2012	GM rice	Pakistan
2	2013.0593	Denmark	26-04-2013	Bt63 rice	Pakistan
3	2012.0252	Denmark	16-02-2012	GM rice	Pakistan
4	2012.0017	France	05-01-2012	GM rice	Pakistan
5	2012.0041	France	10-01-2012	GM rice	Pakistan
6	2012.0018	France	16-01-2012	GM rice	Pakistan

material yielded the result as a single integration, which proves the robustness of the assay developed. It is important to note that this is the first $\Delta\Delta C_T$ based assay to identify *cry1Ac* integrations in rice which could be of use for other GM events involving the same transgene.

It also confirms that the varieties are not a random mixing of transgenic and non-transgenic germplasm in some research project but a deliberate release of GM rice varieties. It is obvious that there is an ongoing practice of cultivating GM rice varieties in the field because randomly selected varieties were analyzed in the screening phase.

Conclusions

The current study basically aimed to verify the application of strict biosafety regulations regarding the production and availability of GM rice in Pakistan. Officially, there is a single transgenic organism (*Bt* cotton – MON531 event) commercialized in the country. According to the biosafety regulations of Pakistan, prior to large-scale cultivation, a GMO must be subjected to a detailed molecular and ecological analysis for horizontal and vertical gene transfer in the ecosystem. Biosafety aspects include a number of issues related to agronomic, nutritional, environmental as well as consumer safety aspects. The cultivation of genetically modified food grains is an even more sensitive issue due to a highly sensitized consumer perception of biological safety dimensions and the potential derivations of the biotechnological products. The results indicate the presence and cultivation of GM rice varieties in the field. A further large scale screening of the commercially grown varieties is urgently required as there could be serious repercussions for the rice export industry if there is a random admixing of conventional and transgenic varieties. For this purpose, the assay established in this study can easily and reliably verify the GM or non-GM status of the rice varieties.

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Detekcja i oszacowanie liczby kopii transgenu *cry1Ac* kodującego cechę odporności na szkodniki w genetycznie modyfikowanym ryżu (*Oryza sativa*)

Streszczenie

Celem niniejszych badań było opracowanie metody detekcji genu *cry1Ac*, odpowiedzialnego za odporność na szkodniki, z wykorzystaniem konwencjonalnej techniki PCR oraz PCR w czasie rzeczywistym, w celu badania obecności tego transgenu w powszechnie uprawianych odmianach ryżu. Detekcja modyfikacji genetycznej obecnej w odmianach ryżu wymaga opracowania

metodyki badawczej z wykorzystaniem metody PCR, zgodnie z ustalonymi procedurami dotyczącymi obecności transgenów w zbożach konsumpcyjnych. Badania te nie tylko umożliwiłyby monitorowanie przepływu transgenów w lokalnym środowisku rolnym, ale również dokonanie charakterystyki różnych gatunków roślin wytworzonych z wykorzystaniem oznaczanego transgenu oraz związanych z nim fragmentów regulatorowych. W niniejszej pracy przedstawiono metodę wiarygodnego testu kontrolnego, w celu detekcji transgenu wraz z promotorem i selekcyjnym genem markerowym. Przy użyciu konwencjonalnej techniki PCR z zastosowaniem starterów specyficznych dla genu *cry1Ac* powielono fragment długości 215 pz. Poszukiwano także, lecz nie wykryto, innych transgenów, takich jak *gna* i *bar*. Obecność genów *p35s*, *sps* i *nptII* została potwierdzona na podstawie techniki ilościowego PCR w czasie rzeczywistym. Poszczególne produkty PCR poddano analizie z wykorzystaniem krzywych topnienia. Ostre wierzchołki krzywych topnienia wskazywały na obecność pojedynczego produktu PCR, odpowiednio dla każdego użytego startera. Za pomocą metody $\Delta\Delta C_T$ oszacowano liczbę kopii genu *cry1Ac*. Na podstawie niniejszych badań wykazano, że zestawy starterów i opracowana metodyka są właściwe do molekularnej detekcji i charakterystyki transgenu *cry1Ac* oraz sekwencji z nim związanych i mogą służyć do wskazania genetycznie modyfikowanych odmian ryżu. Metoda ta może być również pomocna w monitorowaniu środowiska pod względem przepływu tego transgenu.