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DNA markers for phosphine resistance in populations of *Tribolium castaneum* from Punjab, India

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ABSTRACT

Resistance to phosphine fumigant has been reported in several countries including India. A sustainable resistant management strategy calls for sound monitoring systems to avoid impending losses by way of control failures of this fumigant in grain storage godowns, as phosphine is the cost effective fumigant available for protection of grains in bulk storage systems. Although discriminating dose bioassays are used for determination of phosphine resistance levels in insect pests, it would take atleast two weeks to complete these assays. The discovery of genetic variants at the *rph2* locus that alter the function of the dihydrolipoamide dehydrogenase (DLD) gene associated with phosphine resistance resulted in development of DNA based markers for quick detection of phosphine resistance in stored product insect pests such as red flour beetle, *Tribolium castaneum* (Herbst) and lesser grain borer, *Rhizopertha dominica* (Fabricius). In the present study, we have explored the utility of Cleaved Amplified Polymorphic Sequence (CAPS) markers, after phenotypic characterization of resistance to phosphine using discriminating dose assays in nine populations of red flour beetle [*Tribolium castaneum* (Herbst)] collected from wheat (*Triticum aestivum* L.) storage godowns in Punjab state of India. Prevalence of strong resistance to phoshphine was detected in all the tested populations of *Tribolium castaneum* (Herbst) and the molecular diagnostics using CAPS markers have confirmed the prevalence of high percentage of R alleles in the resistant populations of *T. castaneum* in Punjab state of India.

This research also highlighted the utility of DNA markers for understanding the genotype frequency of resistance alleles in *rph2* locus in resistant populations of *T. castaneum* and the possibility of extending this molecular diagnostics tool for monitoring and management of phosphine resistance in *T. castaneum,* a major stored grain pest in India.

Key words: CAPS markers, Phosphine, rph2, Resistance gene, *Tribolium castaneum*

Resistance to the fumigant phosphine has been detected in different stored-product insect pests across the world. Bora and Chahal (1979) first reported the failure of phosphine to control khaprabeetle, (*Trogoderma granarium* Everts) in warehouses in India. Although FAO (1975) bioassays based on discriminating dose of phosphine against a given species are used for detection of resistant individuals in pest insects, the assays require about two weeks for diagnosis of resistance to phosphine in a given insect population.

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The discovery of the gene, dihydrolipoamide dehydrogenase (DLD), a metabolic enzyme associated with phosphine resistance in red flour beetle, [*Tribolium castaneum* (Herbst)] (Schlipalius et al., 2012) and identification of certain number of point mutations conferring strong phosphine resistance have led to the development of a molecular diagnosis technique, Cleaved Amplified Polymorphic Sequence (CAPS) (Kaur et al., 2015), to detect strong phosphine resistance in populations of lesser grain borer, [*Rhyzopertha dominica* (Fabricius)] from Queensland in Australia. Subsequently, the utility of CAPS markers for diagnosis of phosphine resistance has been demonstrated in populations of *T. castaneum* and *R. dominica* from the USA (Chen et al., 2015)

and Turkey (Koçak *et al*., 2015). Considering the annual storage of 60 million tonnes of food grains across India, the development of quick diagnostic tool to monitor the resistance to phosphine in stored product insect pests would help in devising suitable management strategies to manage phosphine resistance in stored insect pests. Here, we present the utility of CAPS markers in detection of phosphine resistance in *T. castaneum* collected from selected wheat storage godowns of Punjab state, India.

MATERIALS AND METHODS

Sample collection

Samples of wheat grains were collected from bulk stored wheat godowns in nine locations in the Punjab state of India during 2012–13 (Fig.1). The grain samples were sieved to separate red flour beetles, which were then cultured on wheat flour at constant regimes of 30ºC and 55% r.h. The bioassays were conducted at the Stored product insect Laboratory of Division of Entomology, ICAR– Indian Agricultural Research Institute, New Delhi, India.

Bioassays

Phenotypic resistance levels were determined for progeny of adults of *T. castaneum* using a modified FAO method (FAO, 1975). Response of field strains to phosphine were examined by phosphine fumigation at low dose (0.03 mg/L) and high dose (0.25 mg/L) at 25±1°C and 70±5% r.h. for 20 h. After fumigation, mortality was assessed following a recovery period of seven days in whole wheat flour at 25±1°C and 55±5% r.h.

Determination of nucleotide variants in the dld *gene*

The protocol followed was the same as described previously by Schlipalius et al. (2012). Total RNA was extracted from 20 individuals of *T. castaneum* using RNA isolation kit (Promega). Complementary DNA (cDNA) synthesis was generated using a SuperscriptIII cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. The coding region of the *T. castaneum dld* gene was amplified from the cDNA with the forward (AGAGGTCACTCGATAATG) and reverse (CGGAAAAAAATGGGCAGC) *rph2* primers using the following PCR conditions: denaturation for 3 min at 95°C, followed by 40 cycles of 95°C for 20 s, 50°C for 30 s and 72°C for 2 min. and a final extension at 72°C for 5 min. The PCR product was visualized using 1% agarose gel with TAE buffer. Following amplification, the DNA fragment was purified and prepared for sequencing using a PCR Purification kit (Qiagen) as per the manufacturer's protocol. Sequencing was done by outsourcing with Scigenome Lab, Cochin, India. Molecular characterization was done to ascertain that the *rph2* genotype was responsible for resistance in test populations collected from Punjab, India.

Extraction of genomic DNA

Genomic DNA was extracted from adult beetles of *T. castaneum* after exposure to discriminating dose bioassays for strong resistance phenotype. Individual adult beetles were placed in cells of a 96-well PCR plate containing 100 µl of TE buffer *p*H 8.0 (10 mM Tris HCl and 1 mM EDTA) and denatured at 98ºC for 20 min, followed by exposure to 25ºC for 5 min and then placed on ice for 5 min . Samples were centrifuged at 5,000 rpm at 4ºC for 15 min and left overnight at 4ºC before use.

CAPS marker assay

A 368 bp fragment of the *dld* gene containing the nucleotide variant corresponding to P45S variant previously reported (Schlipalius et al., 2012) was amplified by PCR (denaturation at 95°C for 3 min followed by 40 cycles of 95°C; 55°C for 20s and 72°C for 30s and a final extension at 7 min.). The amplicons were digested with restriction enzyme MboI to determine resistance genotypes in a cleaved amplified polymorphic sequence (CAPS) marker assay as described by Schlipalius et al. (2012).

RESULTS AND DISCUSSION

Punjab state of northwestern India is the leading food grain producer of the country. It is regarded as a wheat bowl of India and it is one of the top five states producing rice (*Oryza sativa* L.) in India. This state is pivotal to the food security of India, with the storage capacity of 13.5 million tonnes of food grains. Phosphine is the only fumigant used for protecting the grains in bulk storage godowns in the country.

India has a rich history of documentation of resistance to phosphine in stored-product insect pests with the first report on control failure of phosphine was recorded as early as in 1979 (Bora and Chahal, 1979) and a country wide survey during 1990s established widespread occurrence of resistance to phosphine fumigant in stored gram insect pests such as *T. castaneum*, *Sitophilus oryzae* (L.), *R. dominica*, *Oryzaephilus surinamensis* (L.) and *Cryptolestes ferrugineus* (Stephens) (Rajendran, 1992). The present study attempts to validate the utility of CAPS marker developed under AISRF research programme for

Table 1 Genotypes and corresponding frequency of the resistant allele as detected by CAPS markers for *rph2*

Populations	Resistance phenotype	N	RR	RS	SS	% R alleles	% S alleles
Bhatinda	Strong	20	15	4	1	85.00	15.00
Ferozpur	Strong	30	3	27	0	55.00	45.00
Gurdaspur	Strong	30	2	28	0	53.33	46.67
Patiala	Strong	30	3	27	0	55.00	45.00
Pansup Sarhind	Strong	30	8	22	0	63.33	36.67
Sangrur	Strong	28	8	20	0	64.29	35.71
Jalandar	Strong	28	18	10	0	82.15	17.86
Hoshiarpur	Strong	28	6	18	4	53.57	46.43
Ludhiana	Strong	30	17	12	1	76.67	23.33

N, Number of adult beetles used for genotype analysis. The frequencies of resistant alleles for *rph2* were determined for each population of *T. castaneum*after exposure to FAO discriminating dose assays for strong resistance

Fig. 1. Locations of field populations of *T.castaneum* from Punjab state, India. 1, Bhatinda; 2, Ferozpur; 3, Gurdaspur; 4, Patiala; 5, Pansup Sarhind; 6, Sangrur; 7, Jalandhar; 8, Hoshiarpur, 9, Ludhiana

detection of phosphine resistance in red flour beetle, (*T. castaneum*).

The resistant phenotypes were evaluated using discriminating dose bioassays for weak resistance (0.03 mg/L) and strong resistance (0.25 mg/L). The survival of the beetles were 91–100% in weak resistance bio assays and it was 53–100% in the strong resistance assays implying that all the populations had strong resistance phenotype (Table 1).

Individual genotypes and population allele frequencies derived from the MboI-CAPS markers with bioassay characterized resistance frequencies for nine populations of *T. castaneum* are furnished in Table 1. The genotype allele frequencies (Fig. 2) revealed that the R alleles were ranging from 53 to 85% with *T. castaneum* populations collected from Jalandhar and Batinda were recording high proportion of rr alleles indicating that populations are evolving to become homozygous for resistance to phosphine (Fig. 3). Both these populations which were recording the highest proportion of R alleles were found to

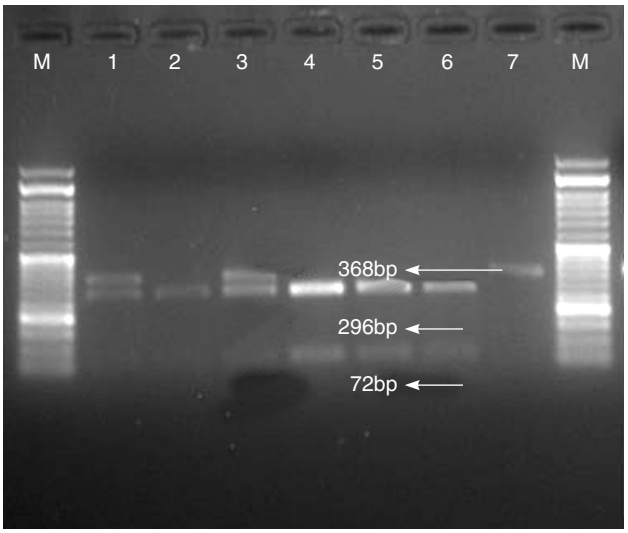

Fig. 2. A representative gel on demonstration of utility of CAPS markers in *T. castaneum* individuals from Jalandhar, Punjab, India. PCR amplicons genomic DNA coding for the DLD gene were digested with restriction enzyme, *MboI*. Lanes M on extremes: 100 bp DNA ladder; Lanes 1–7 on gel are digested products from homozygous resistant (RR) - 296 bp and 72 bp; susceptible SS - 368 bp and heterozygous (RS) – 368 bp and 296 bp

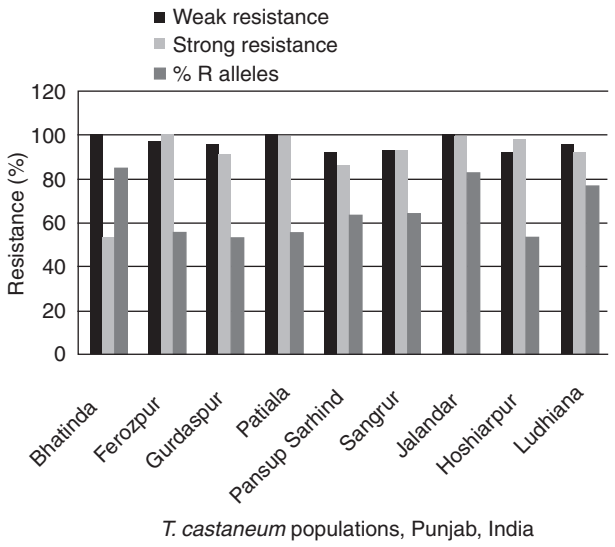

Fig. 3. Frequency of phosphine resistant phenotypes in nine populations of *T. castaneum*, collected in Punjab state, India. Phenotypes were determined using discriminating dose bioassays for weak resistance (30 ppm phosphine for 20 h) and strong resistance (180 ppm for 20 h). The per cent surviving the bioassay for a givenpopulation is reported here. Also shown are the CAPS results for the frequency of the strong resistance (R) allele ineach population

have the highest resistance frequency (>90 %) (data from unpublished results) in the strong resistance bioassay. Thus the CAPS markers have provided valid information for determining the genotype and help predict the evolution of strong resistance in a pest population. Our results confirm the validity of the CAPS markers in genotype analysis of phosphine resistance in *rph2* locus as earlier documented with *R. domininca* populations from Australia (Kaur et al., 2013), and both *R. dominica* and *T. castaneum* populations from the USA (Chen et al., 2015). The CAPS markers have been proved to be efficient in detection of strong resistance genotypes in Indian *T. castaneum* populations.

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