



Molecular markers of *Ry_{adg}* gene and serological assay reveal potato virus Y (PVY) resistance in the tetraploid Indian potato (*Solanum tuberosum*) germplasm

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Received: 02 July 2011; Revised accepted: 5 February 2013

ABSTRACT

The *Ry_{adg}* gene derived from *Solanum tuberosum* ssp *andigena* provides extreme resistance to PVY. The PVY resistant potato germplasms tested previously through ELISA were used in present experiment for examining presence/absence of *Ry_{adg}* gene through linked molecular markers. SCAR and CAPS markers were used as a tool for selecting resistant clones in early generation, to determine the source of resistance and validate the earlier results. Fifty four potato germplasms were screened with molecular markers and by ELISA after mechanical inoculation with PVY^O isolate. Eight germplasms validated PVY resistance by containing *Ry_{adg}* gene and negative absorbance value ($A_{405\text{ nm}}$) of ELISA as well. On the contrary, 39 germplasms were found resistant through ELISA but did not show *Ry_{adg}* gene. Therefore these 39 resistant germplasms would be the future research materials to identify source of resistance through different molecular markers from wider genetic base of wild potatoes species. Thus, our results demonstrate the application of *Ry_{adg}* gene linked molecular markers as early, rapid and more reliable technique for the screening of PVY resistance in potato germplasms as an alternative to ELISA.

Key words: ELISA, Molecular markers, PVY resistance, *Ry_{adg}* gene, *Solanum tuberosum*

Potato virus Y (PVY, *Potyvirus*) is the most damaging potato virus that causes severe yield reduction up to 80% alone or in combination of other viruses, depending upon cultivars, time of infection and environment. Once the plant gets infected, the virus spreads systemically throughout the plant-parts making it difficult to eradicate from seed (tuber) lots. The vegetative mode of propagation of potato enables viruses to persist from one generation to others resulting in continuous decline in productivity which is known as clonal degeneration (Khurana 1999). Breeding PVY resistant cultivars through introgression of resistance (*Ry*) gene is the best strategy to overcome this problem. For selection of PVY resistant germplasms/clones, use of molecular markers tightly linked to *Ry* gene- for example the *Ry_{adg}* gene conferring extreme resistance to PVY- may enhance reliability and efficiency of breeding procedure.

Unlike the race specific *Ny* genes, the *Ry_{adg}* gene of *S.*

tuberosum ssp *andigena* ($2n = 4x = 48$) confers race non-specific durable resistance to PVY (Ross 1986, Mihovilovich *et al.* 1998). The property of extreme resistance and race non-specificity of the *Ry_{adg}* gene have made it an ideal source of PVY resistance for the potato breeding programs. This gene has been genetically mapped to chromosome XI (Hämäläinen *et al.* 1997) and linked PCR-based DNA markers have been developed. Molecular markers, viz. SCAR (Sequence characterized amplified region) and CAPS (Cleaved amplified polymorphic sequence) tightly linked to *Ry_{adg}* gene have been developed by Kasai *et al.* (2000) and Sorri *et al.* (1999), respectively. Selection of resistant parental lines using these markers is a useful pre-breeding technique in breeding programs for virus resistance. Marker assisted selection will save time and other resources that are needed for evaluating large segregating population for several generations. This indirect selection is expected to be more effective than direct selection due to the absence of confounding effects of the environment.

In an effort to maximize selection efficiency for the genotypes resistant to PVY, present research work was conducted on validation and application of SCARs and CAPS molecular markers for the selection of PVY resistant

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germplasm lines containing *Ry_{adg}* gene. The result of virus screening of the same genotypes by mechanical inoculation followed by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) for testing virus multiplication *in vivo* has also been discussed here.

MATERIALS AND METHODS

Plant materials used in this study included 54 tetraploid potato germplasms available in the repository of Division of Crop Improvement, Central Potato Research Institute (CPRI), Shimla, Himachal Pradesh, India. These germplasms have been reported to be resistant to PVY^O infection based on earlier screening experiments done at CPRI, Shimla, Himachal Pradesh (Anonymous 2002 to 2008). These germplasms (3 tubers per line) were grown in earthen pots filled with the mixture of sieved-soil, farmyard manures and sands (2:1:1) during summer season (April-August) of 2009 in the glass house at CPRI, Shimla. Plants were watered regularly and fertilized to maintain good health. An advance hybrid clone LBY-26 was used as a positive clone for PVY resistance, whereas, commercial potato cv Kufri Jyoti developed by CPRI, Shimla was used as PVY susceptible check in this study.

Plants were mechanically inoculated using PVY^O isolates maintained in tobacco (*Nicotiana glutinosa* L. and *N. tabacum* L.) plants at CPRI, Shimla. Infected fresh tobacco leaf tissue (~ 2.5 g) was ground in 25 ml of cold 1 mM potassium phosphate (pH 8) buffer. Two to three young leaves of each plant were dusted with carborandrum powder and were then lightly rubbed with cheesecloth dipped in virus inoculum. Plants were evaluated at 20 days after inoculation for PVY expression and tested by das-ELISA.

PVY^O titers were determined using ELISA with PVY^O antibodies developed at CPRI, Shimla. Fresh tissue from the third compound leaf (from the top) of artificially inoculated plants was collected and ground in a buffer at a ratio of 1:10 (50 mg tissue: 500 µL grinding buffer) using a hand crushing machine. ELISA was conducted following the method described by International Potato Centre, Lima-Peru (Anonymous 2007). Two negative and two positive controls maintained at CPRI, Shimla were used in each 96-well ELISA plate. After incubating for one hour, absorbance values were measured at 405 nm (A_{405} nm) using an ELISA reader (BioTek, Winooski VT, USA). The absorbance threshold for susceptible genotypes was set at absorbance levels two times greater than the mean for negative controls (Sutula *et al.* 1986).

Genomic DNA was isolated using leaf samples collected in Liquid Nitrogen (-196 °C) from glass-house grown plants according to the modified CTAB method of Saghai-Marooof (1984). Leaves (~ 300 mg) were ground manually in mortar-pestle in 1 ml CTAB extraction buffer. Crushed suspension was added with 1 ml dichloromethane and incubated at 65°C for 1 hr followed by centrifugation at maximum speed for 15

min. Supernatants were taken in fresh 1.5 ml centrifuge tubes and 0.5 ml 2-propanol was added and centrifuged as above. White DNA pellets were resuspended in absolute ethanol and incubated overnight at -20°C. The suspension was then centrifuged at maximum speed (14000 rpm) (Sigma Aldrich, Saint Louis MO, USA) followed by washing with 70% ethanol and dried pellets were dissolved in T₁₀E₁ buffer (100 µl). This DNA was treated with 1 µl RNase (10 mg/ml) followed by precipitation with 2-propanol and ethanol washing as above. DNA quantification and quality ($A_{260}/A_{280}=1.8-2.0$) was measured using Spectrophotometer UV-1700 (Shimadzu, Kyoto, Japan) and further used in PCR analysis.

PCR amplification of markers linked to *Ry_{adg}* was carried out using a Mastercycler Gradient (Eppendorf, Hamburg, Germany) with SCAR and CAPS primers originally developed by Kasai *et al.* (2000) and Sorri *et al.* (1999), respectively. The SCAR markers identified as RYSC3 and RYSC4, and the CAPS marker ADG2/*BbvI* are tightly linked and show a high degree of accuracy for the detection of *Ry_{adg}* gene. Each PCR reaction mixture contained 1X PCR buffer, 2.5 mM MgCl₂, 200 µM dNTPs, 0.2 µM of each primer, 1 Unit Taq Polymerase (Qiagen) and 50 ng template DNA for final reaction volume of 20 µl. The PCR program for the SCAR markers (Kasai *et al.* 2000) consisted of an initial denaturation step at 94°C for 5 min., followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis in a 1.6% agarose gel in 0.5X TBE (Tris-borate-EDTA, pH 8) using horizontal gel electrophoresis system Sub-Cell GT (Bio-Rad, USA) at room temperature. The gels were visualized under Gel Doc System (Alpha Innotech, San Leandro, CA, USA) and compared with 100 bp DNA ladder (Fermentas, Burlington, Canada). Presence of a 321 base pair (bp) band for RYSC3, and presence of a 145 bp band for RYSC4 was associated with PVY resistance gene *Ry_{adg}*. Absence of the amplified products was associated with susceptibility to PVY (Kasai *et al.* 2000).

The PCR program for the CAPS marker (Sorri *et al.* 1999) consisted of an initial denaturation step at 93°C for 2 min., followed by 40 cycles of denaturation at 93°C for 45 s, primer annealing at 45°C for 45 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 5 min. The PCR products of ADG2 were digested in a reaction volume of 20 µl with 0.05 U/µl *BbvI* enzyme, 1X enzyme buffer (NEB2). Samples were digested at 65°C for 3 hours and products were then visualized on a 1.6% agarose gel in 1X TBE. Presence of an undigested product of 355 bp was associated with the PVY resistance gene *Ry_{adg}*. Presence of one digested product of 270 bp was associated with PVY susceptibility (Sorri *et al.* 1999). Final selection was based on the association of markers with the PVY resistance gene *Ry_{adg}*.

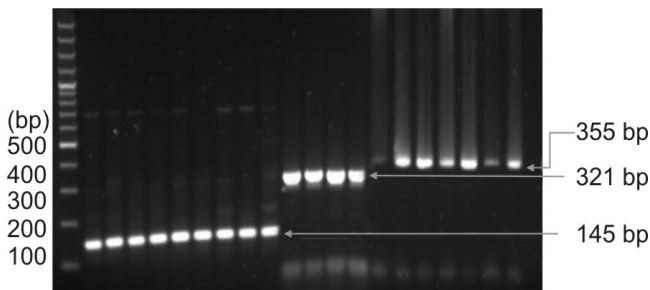


Fig 1 DNA amplification of SCAR markers RYSC4 and RYSC3 amplified at 145 bp and 321 bp, respectively whereas CAPS markers ADG2/BbvI amplified at 355 bp in the PVY resistant potato germplasms.

Statistical analysis

Correlation coefficient between the ELISA results based on absorbance value (A_{405} nm) and the presence/absence of Ry_{adg} gene through molecular markers for PVY resistant lines was analyzed using SYSTAT Software (Systat Software, Inc. Washington).

RESULTS AND DISCUSSION

In the present study, a total of 54 potato germplasm lines were evaluated for PVY resistance through mechanical inoculation followed by ELISA screening. The presence/absence of the Ry_{adg} gene in those lines was also checked by using tightly linked SCARs (RYSC3 and RYSC4) and CAPS (ADG2/BbvI) markers. In the pathogenicity study, 47 lines out of 54 showed resistant response while 7 lines (CP Nos. 1974, 1988, 2400, 2930, 3261, 3413 and 3451) were susceptible to PVY when tested by mechanical inoculation and screening by ELISA after 20 days of inoculation. The ELISA readings (A_{405} nm) of susceptible lines after 20 days of mechanical inoculation were more than double (> 0.05) from that of the negative healthy control (0.025). These 7 susceptible lines had been identified as resistant to PVY infection in earlier screening experiments. In molecular analysis, none of the 7 susceptible lines showed Ry_{adg} marker allele as well, suggesting that most probably they were erroneously identified as PVY resistant in earlier studies due to problem in pathogen inoculation and screening which is a common error in biotic interaction studies. It suggested that before using a particular germplasm line in breeding project for virus resistance, it is necessary to evaluate it both by PVY infection followed by different types of molecular markers linked to the resistant phenotype. In case of other 47 lines, pathogen screening result corroborated earlier data (Table 1).

The tetraploid potato germplasms used in the study were evaluated for the presence/absence of Ry_{adg} gene through SCARs and CAPS markers as well. Marker analysis indicated the presence of linked alleles associated with Ry_{adg} gene in 8 out of 47 PVY resistant germplasms lines (CP Nos. 1881, 1970, 2146, 2297, 2383, 2401, 3414 and 3421). All eight

lines were positive for both RYSC3 (321 bp), and RYSC4 (145 bp) SCAR markers (Kasai *et al.* 2000) as well as ADG2/BbvI CAPS marker (Sorri *et al.* 1999). These lines had been scored as PVY resistant during at least two years of virus screening. The present screening study, therefore, confirmed the earlier findings and also demonstrated that the source of PVY resistance in those 8 germplasm lines has been derived from *S. tuberosum* spp *andigena*. The correlation co-efficient between ELISA readings and molecular markers was only 16.09% suggesting no correlation between PVY resistance and presence/absence of linked marker within the population of germplasm studied in the present study. This happened because some resistant lines (39 in numbers) obtained through ELISA screening did not show markers linked with the Ry_{adg} gene. It suggested that PVY resistance in those 39 lines was derived from sources other than *S. tuberosum* spp *andigena*. These lines, therefore, would be valuable research material for future validation of markers linked with other PVY resistant genes derived from both wild and cultivated potato species. The present study confirmed PVY resistance in 8 germplasm accessions and also identified the resistant gene as Ry_{adg} through molecular marker analysis. The markers validated in the present study can be used for marker assisted selection of breeding population derived from the crosses involving any of the 8 germplasm lines as parent. Our findings corroborated the strategy followed with Jonathan *et al.* (2009) and Ryon *et al.* (2009) for the implementation and validation of Ry_{adg} molecular markers in potato breeding through marker-assisted selection (MAS).

The practical application of molecular markers developed for the mapped genes in potato breeding programs has been progressing at a slower speed in the India and abroad. Published examples are mainly limited to diploid material and a small number of genes, ie *Rx1* for extreme resistance to PVX (Ritter *et al.* 1991), Ry_{adg} for extreme resistance to PVY (Sorri *et al.* 1999, Kasai *et al.* 2000), *Ns* for PVS resistance (Marczewski *et al.* 2002), *Gro1* for resistance to *G. rostochiensis* (Paal *et al.* 2004) and *Sen1* for resistance to potato wart (Gebhardt *et al.* 2006). Though molecular markers have been identified for PVY resistance from *S. stoloniferum* (Song *et al.* 2005, Valkonen *et al.* 2008) and *S. tuberosum* ssp. *andigena* (Sorri *et al.* 1999, Kasai *et al.* 2000, Rizza *et al.* 2006, Valkonen *et al.* 2008) their application in breeding program is still limited to few instances. On the contrary, the SCAR and CAPS markers linked to the Ry_{adg} gene have been used in a comparatively wider scale in breeding program for virus resistance. The ability to check for the presence of Ry_{adg} gene through molecular markers before hybridizing parents or before the field screening of progeny can increase selection efficiency for PVY resistance in potato breeding. Before relying on a specific set of markers in a breeding program, it would be important to test the markers in germplasm with known resistant species background and to confirm their resistance responses to the various PVY strains.

Table 1 Markers showing presence/absence of *Ry_{adg}* gene conferring extreme resistance to PVY and response to PVY infection through artificial inoculation in the potato germplasm

Germplasm (CP No.)	PVY (Resistance/ Susceptible)							Molecular markers (Presence/Absence)		
	2002-03	2003-04	2004-05	2006-07	2007-08	2008-09	2009-10	RYSC3	RYSC4	ADG2/Bbv I
CP-1597	na	na	na	na	R	na	R	-	-	-
CP-1881	na	na	na	na	R	na	R	+	+	+
CP-1917	R	na	na	na	na	na	R	-	-	-
CP-1970	R	R	na	na	na	na	R	+	+	+
CP-1971	R	R	na	na	R	na	R	-	-	-
CP-1974	na	R	na	na	na	na	S	-	-	-
CP-1978	na	na	na	R	na	na	R	-	-	-
CP-1987	R	na	na	R	na	na	R	-	-	-
CP-1988	na	na	R	na	R	na	S	-	-	-
CP-1990	na	na	na	R	R	na	R	-	-	-
CP-1996	R	R	na	na	R	na	R	-	-	-
CP-2133	na	na	na	na	R	na	R	-	-	-
CP-2146	na	na	na	R	na	na	R	+	+	+
CP-2217	na	na	na	na	R	R	R	-	-	-
CP-2246	na	na	na	R	R	na	R	-	-	-
CP-2289	na	na	na	na	R	na	R	-	-	-
CP-2297	na	na	na	R	R	na	R	+	+	+
CP-2304	na	na	na	R	R	na	R	-	-	-
CP-2312	na	na	na	R	R	na	R	-	-	-
CP-2316	na	na	na	R	R	na	R	-	-	-
CP-2322	na	na	na	R	R	na	R	-	-	-
CP-2336	na	na	na	R	R	na	R	-	-	-
CP-2350	na	na	na	R	R	na	R	-	-	-
CP-2358	na	na	na	R	R	na	R	-	-	-
CP-2364	na	na	na	R	R	na	R	-	-	-
CP-2369	na	na	na	R	R	na	R	-	-	-
CP-2376	na	na	na	R	R	na	R	-	-	-
CP-2379	na	R	na	R	R	na	R	-	-	-
CP-2383	na	na	na	R	R	na	R	+	+	+
CP-2384	na	na	na	na	R	na	R	-	-	-
CP-2400	na	na	na	R	R	na	S	-	-	-
CP-2401	na	na	na	R	R	na	R	+	+	+
CP-2428	na	na	na	R	R	na	R	-	-	-
CP-2930	na	na	na	R	R	na	S	-	-	-
CP-3068	na	na	na	na	R	na	R	-	-	-
CP-3091	na	na	na	na	R	na	R	-	-	-
CP-3093	na	na	na	na	R	na	R	-	-	-
CP-3146	na	na	na	na	R	R	R	-	-	-
CP-3159	na	na	na	na	R	na	R	-	-	-
CP-3171	na	na	na	na	R	na	R	-	-	-
CP-3175	na	na	na	na	R	na	R	-	-	-
CP-3187	na	na	na	na	R	na	R	-	-	-
CP-3198	na	na	na	na	R	na	R	-	-	-
CP-3247	na	na	na	na	R	na	R	-	-	-
CP-3261	na	na	na	R	R	na	S	-	-	-
CP-3262	R	na	na	na	R	na	R	-	-	-
CP-3413	na	na	na	na	R	na	S	-	-	-
CP-3414	na	na	na	na	R	na	R	+	+	+
CP-3421	na	na	na	na	R	R	R	+	+	+
CP-3443	na	na	na	na	R	R	R	-	-	-
CP-3451	na	na	na	na	R	na	S	-	-	-
CP-3533	na	na	na	na	R	R	R	-	-	-
CP-3534	R	R	na	na	R	na	R	-	-	-
CP-3762	na	na	na	na	R	R	R	-	-	-

R/S : Resistance/Susceptible; +/- : presence/absence; na not available

In conclusion, the molecular markers linked to *Ry_{adg}* proved informative in identifying resistant individuals within a group of parental lines for extreme resistance to PVY. This finding supports the use of molecular markers for MAS in potato breeding program to accelerate the development of PVY resistant cultivars. In addition, a survey of diverse breeding clones and cultivars using the molecular markers was useful in determining whether observed PVY resistance was conferred by *Ry_{adg}* or by other resistance genes. Nevertheless, traditional screening methods are still needed in the identification of PVY resistant germplasm with resistance conferred by genes different from tagged gene like *Ry_{adg}* and *Ry_{sto}*. This will help potato breeders in the development of durable resistance to PVY by the pyramiding of divergent resistance genes. In future, as more virus resistance loci are being tagged, it is expected that the application of molecular markers in the development of new potato cultivars will increase the importance of MAS.

ACKNOWLEDGEMENTS

Authors thank to the Indian Council of Agricultural Research, New Delhi for providing financial support for successful completion of the research work. The services rendered by Mr Rajinder for ELISA testing and Mr Sheeshram Thakur for maintenance of plants in the glasshouse are gratefully acknowledged. Heartly thanks are extended to Dr S K Kaushik for providing an advance PVY resistant hybrid clone.

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