

Identification of elite potato genotypes possessing multiple disease resistance genes through molecular approaches



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ABSTRACT

World potato productivity including India was almost stagnant during the last two decades largely due to yield losses by biotic and abiotic stresses. Among biotic stresses, late blight, viruses and nematodes are the most devastating. Varieties resistant to individual stress have been deployed but the production remained limited because of other biotic stresses affecting the crop. Potato cultivars having multiple disease resistance are urgently required to boost production. Resistance to late blight is both qualitative and quantitative while extreme resistance to PVY can be imparted by the single dominant genes *Ry_{adg}* and *Ry_{sto}*. Likewise resistance to potato cyst nematode is mainly imparted by the single dominant *H1* and *Gro1-4* genes. All these genes have been mapped and tightly linked molecular markers are available to perform marker-assisted selection (MAS). In the present study 126 parental clones were characterized for the presence of genes for resistance to late blight (*R1*, *R2*, *R3a*), PVY (*Ry_{adg}*, *Ry_{sto}*) and potato cyst nematodes (*H1*, *HC_QRL* and *Gro1-4*) using molecular markers. The same clones were evaluated for disease resistance with standard phenotypic assays. Fourteen elite potato genotypes possessing multiple disease resistance genes were identified by means of linked molecular markers and their resistances were confirmed through phenotypic screening methods. These genotypes can be exploited as parents for hybridization to expedite the potato resistance breeding programmes.

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1. Introduction

Potato (*Solanum tuberosum* L.) is a member of the Solanaceae, an economically important plant family. Potato is after the grains, the world's number one food crop with global production reaching a record of 374 million tonnes in 2011 (<http://faostat.fao.org/site/339/default.aspx>). Today, potatoes are grown in about 150 countries ranging from latitudes 23° N to 57° N and at altitudes from sea level to 4000 amsl. The top three potato producers of the world, China, India and the Russian Federation together contribute about 43% of the global potato production. A yield plateau has been reached for the potato

crop in India (Pandit and Chandran, 2011) indicating that the potato varieties developed during the last 50 years did not find substantial favour with the growers. The world potato productivity also did not substantially increase during the last 20 years, though an increase from 14.6 t/ha (1991) to 17.8 t/ha (2010) was observed (<http://faostat.fao.org/site/339/default.aspx>). Gopal and Oyama (2005) stated that stagnation of the potential yield of potato commercial cultivars is primarily due to their narrow genetic base. Hence, to ensure food security, buffer stocking and exportable surplus, high yielding, multiple disease resistant potato varieties are required (Douglas and Halpin, 2010; Joseph et al., 2011).

Among the various biotic and abiotic stresses affecting potato production, three biotic stresses i.e. late blight, viruses and cyst nematodes constitute major threats. Late blight caused by the oomycete, *Phytophthora infestans* is the most important disease of potato causing severe crop damage worldwide. It causes losses over US \$ 3.25 billion in developing countries while in India, it inflicts

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losses up to 15% annually amounting to 0.5 billion US \$ (Bhat et al., 2008). Since early in the last century 11 single genes for resistance (*R* genes) to late blight have been introgressed into the cultivated potato from *S. demissum*. This type of resistance, often called vertical resistance, was rapidly overcome in the field by new strains of *P. infestans*, and thus breeders lost interest in single gene resistance (Wastie, 1991). Now-a-days there is renewed interest in this type of resistance as *R* genes, although defeated, can still increase the level of quantitative or field resistance, which is considered more durable (Stewart et al., 2003; Gebhardt et al., 2004). Co-localization of some *R* genes with quantitative resistance loci (QRL) by Gebhardt and Valkonen (2001) suggests that major and minor gene resistance might be in part similar at the molecular level. Most of the *R* genes introgressed from *S. demissum* have been mapped and four of them *R1*, *R2*, *R3a* and *R3b* have been cloned and sequenced (Ballvora et al., 2002; Lokossou et al., 2009; Huang et al., 2005; Li et al., 2011). Many other *R* genes conferring resistance against *P. infestans* have been mapped from wild species including *RB/Rpitbl1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* and *Rpi-abpt* (*S. bulbocastanum*); *Rpi-bt1* (*S. brachistotrichum*); *Rpi-edn1.1* (*S. edinense*); *Rpi-hjt1.1*, *Rpi-hjt1.2* and *Rpi-hjt1.3* (*S. hirtetingii*); *Rpi-mcd1* (*S. microdontum*); *Rpi-sn1.1* and *Rpi-sn1.2* (*S. schenckii*); *Rpi-ver1* (*S. verrucosum*); *Rpi-pnt1* (*S. pinnatisectum*); *Rpi-sto1* and *Rpi-sto2* (*S. stoloniferum*); *Rpi-pt1* (*S. papita*); *Rpi-plt1* (*S. polytrichon*); *Rpi-mcq1* from *S. mochiquense*; *Rpi-phu1* from *S. phureja*; *Rpi-vnt1.1*, *Rpi-vnt1.2*, *Rpi-vnt1.3* (*S. venturii*); *Rpi-dlc1* from (*S. dulcamara*); *Rpi-ber1* and *Rpi-ber2* (*S. berthaultii*); *Rpi-avl1* (*S. avilesi*); *Rpi-cap1* (*S. capsicibaccatum*); and *Rpi-qum1* *S. circaeifolium* spp. *quimense*. Among these about 20 functional late blight *R* genes have also been cloned so far (Kim et al., 2012). As a result of this work molecular markers either tightly linked to these genes or located within the resistance gene itself have been developed, which can be useful for undertaking marker assisted selection (MAS).

The potato crop is also threatened by a number of viruses which are omnipresent (Jeffries, 1998). The most damaging viral diseases are caused by Potato Leaf Roll Virus (PLRV), Potato Virus Y (PVY) and Potato Virus X (PVX). Currently, PVY causes the most damage and surpassed PLRV in numbers, with yield losses up to 80% (Daniels and Pereira, 2004). Three main strains of PVY are known to infect a wide range of potato cultivars viz., PVY^C, PVY^N and PVY^O, though further PVY strains have emerged due to mutation or recombination among these strains, resulting in more aggressive strains like PVY^{NTN}. The symptoms produced by PVY in potato foliage vary according to the virus strain and the cultivar. PVY^C causes a mosaic pattern while PVY^N leads to leaf necrosis in tobacco and soft mosaic symptoms in potato foliage. Necrotic rings are produced by PVY^{NTN} and severe necrosis can lead to death of the susceptible plants. PVY^O is the most common strain in India, causing light and dark mosaic patterns in the leaves. Further spreading of the virus may lead to vein burning and sometimes necrotic "ringlets" on the leaves.

In India more than 90% of potatoes are grown under sub-tropical conditions that favour the proliferation of viruses because of the congenial conditions prevalent for their insect vectors. This fact makes it difficult to produce virus free healthy seed potatoes. The situation is expected to deteriorate further due to global warming that may result in fewer days available for seed potato production. Hence, breeding virus resistant cultivars as advocated by Ross (1986) needs to be taken up on priority to boost the seed production programmes in the developing world. The strain-specific resistance is controlled by the *N_Y* resistance gene while the extreme resistance is controlled by *R_Y* gene. The single dominant gene *Ry_{adg}* from *S. tuberosum* group *andigena* on potato chromosome XI imparts extreme resistance (ER) to PVY (Munoz et al., 1975). The *Ry_{adg}* gene can be detected in segregating progenies with the diagnostic DNA marker RYSC3 (Kasai et al., 2000). Recently CPRI

has developed and registered the elite potato cultivar YY6/3 C-11 with the National Bureau of Plant Genetic Resources (NBPGR), New Delhi (INGR10143). This cultivar possesses *Ry_{adg}* in triplex (YYY) condition (Kaushik et al., 2013). The independent gene *Rysto* on chromosome XII from *S. stoloniferum* also confers extreme resistance to PVY and can be diagnosed by PCR markers (Flis et al., 2005; Song et al., 2005; Song and Schwarzfischer, 2008). Other sources of resistance genes to PVY are detected in wild species of *S. chacoense* (*Ry_{chc}*, *Ny_{chc}*), *S. hougasii* (*Ry_{hou}*), *S. demissum* (*Ny_{dms}*) by Cockerham (1970) and Valkonen et al. (1994). Two other novel genes *Ny_{tbr}* and *Ny-1* were identified in *S. tuberosum* Gp. Chilotanum by Celebi-Toprak et al. (2002) and Szajko et al. (2008) respectively.

The potato cyst nematodes (PCN) *Globodera rostochiensis* and *Globodera pallida* are the most important pests feeding on potato roots (Evans and Trudgill, 1992). The symptoms of infection are unspecific and similar to those caused by other biotic or abiotic stresses. Yield losses caused by PCN are estimated up to 30% worldwide (Oerke et al., 1994). Both PCN species are included in the list of quarantine pathogens in many countries (EPPO/CABI, 1992) including India. The wild *Solanum* species mostly exploited in PCN resistance breeding are *S. tuberosum* ssp. *andigena*, *S. vernei* and *S. spegazzini*. PCN resistance has been reported in other wild sources viz., *S. gourlayi*, *S. sparsipilum*, *S. chacoense*, *S. phureja*, *S. demissum*, *S. gourlayi*, *S. microdontum*, *S. sucrensis*, *S. tarijense*, *S. acaule*, *S. fenderi*, *S. multidissectum*, *S. oplocense* (Phillips, 1994). A number of PCN resistance genes have been mapped in different potato chromosomes conferring specific and partial resistance. Major genes conferring specific resistance to *G. rostochiensis* are *H1*, *GroVI*, *GroI*, *GroI-4* while *Gpa2*, *GpaV* and *GpaXI* confer resistance against *G. pallida* (Milczarek et al., 2011) Besides, several other major and minor QTL offers partial resistance to either of these *Globodera* species. The phenotypic evaluation of resistance to *Globodera* spp. is costly and time consuming. DNA markers can reduce these costs, when their application in breeding programmes is optimized, for example by consecutive screening or by multiplex PCR assays (Gebhardt et al., 2006). Currently available diagnostic markers for the selection of PCN resistant genotypes (Milczarek et al., 2011; Dalamu et al., 2012) are TG689 (Biryukova et al., 2008), Gro1-4 and Gro 1-4-1 (Paal et al., 2004; Gebhardt et al., 2006; Asano et al., 2012), SPUD1636 (Bryan et al., 2002) and the SNP-based marker HC (Sattarzadeh et al., 2006).

In order to increase and sustain potato production, it is imperative to control these diseases effectively. Chemical control of any of these diseases has its own limitations. Therefore, exploiting genetic resistance was and is always the advised and preferred method for their effective management. Molecular markers tightly linked to different resistance genes have the potential to facilitate the precise and efficient selection of resistant cultivars at an early stage, particularly the stacking of two or more different resistance genes in the same genetic background. The availability of DNA-based markers closely linked to resistance genes/QRLs offers the possibility to screen various types of germplasm. Stacking genes for resistance to late blight and nematodes through this approach will extend the life span of new varieties in the South Indian hill regions while varieties combining resistance to late blight and viruses will have great potential in the sub-tropical plains of India. Developing varieties with diverse resistance genes and their strategic deployment in different agro-climatic zones would help in arresting the spread of major diseases in different potato growing areas. Keeping this in view, the objectives of the present study were (i) to identify parental potato lines possessing multiple disease resistance genes to be exploited in potato breeding programmes and (ii) to test the suitability for MAS of several diagnostic markers for disease resistance in Indian breeding materials.

2. Materials and methods

2.1. Plant materials

The plant material screened for resistance and presence of markers linked to late blight, PVY and PCN resistance genes comprised 126 potato genotypes maintained at the National Germplasm Repository at Shimla (HP), India. This collection included exotic potato germplasm accessions (52) imported from 10 different countries, which are currently used as parental lines, Indian potato varieties (44) and advanced potato hybrids (30). Besides *S. demissum* derived set of 22 late blight differentials consisting of 11 major late blight genes ([Malcolmson and Black, 1966](#)) and combination of these genes, developed by International Potato Centre with known *R* gene background was used to validate the *R* genes.

2.2. PCR assay

Details of the markers used are shown in [Table 1](#). PCR was performed in 20 µl reaction buffer (10 mM Tris-HCl pH 9.0, 50 mM KCl, 15 mM MgCl₂), containing 0.125 mM of each dNTP, 0.5 µl of 5 µM of each primer and 0.3 µl of 3.0 units/µl of Taq DNA polymerase (Bangalore Genei, Peenya, KA, India) and 100 ng template

DNA. Amplification reactions were carried out in a C-1000 Thermal Cycler (Bio-Rad, CA, USA). The amplified fragments were resolved on 1.5–2.5% agarose gel depending on the size of the specific fragment, using a horizontal gel electrophoresis system (Bio-Rad, CA, USA).

2.3. Phenotyping assays

To confirm the genotypic results, we evaluated all the 126 genotypes for the phenotypic resistance response towards late blight, PVY and PCN infections.

2.3.1. Screening for late blight resistance

Foliage resistance to late blight was assessed in the laboratory by the detached leaf method. Forty-five days old plants were used for disease assessment. The fourth leaf from the top was plucked from each plant in five replicates, placed in plastic trays on perforated plastic separators ([Umaerus and Lihnell, 1976](#)) and drop inoculated with a *P. infestans* zoospore suspension (~40,000 sporangial zoospores/ml). The inoculum was a complex *P. infestans* race maintained at CPRI (Kalyani-08-1 and HP-10-45), which overcomes most *R1–R11* genes. The inoculated leaves were incubated under high humidity (>90%) at 18 ± 1 °C for 6 days and then the lesion area

Table 1
Details of the markers used for germplasm screening.

Marker	Gene	Type ^a	PCR conditions	Size (bp)	Primer Sequence (5'-3')	References
Late blight R1AS	<i>R1</i>	AS	94 °C, 180 s; 35 × (94 °C, 35 s; 60 °C, 45 s; 72 °C, 90 s); 72 °C, 180 s	1400	F-CACTCGTGACATATCCTCACTA R-CAACCCTGGCATGCCACG	Ballvora et al. (2002)
CosA	<i>R1</i>	SCAR	94 °C, 120 s; 35 × (94 °C, 20 s; 55 °C, 20 s; 72 °C, 30 s); 72 °C, 300 s	210/250	F-CTCATTCAAATCAGTTTGAC R-GAATGTGAATCTTTTGTGAAG	Gebhardt et al. (2004)
R2	<i>Rpi-abpt</i> (R2-Ortholog)	SCAR	94 °C, 300 s; 35 × (94 °C, 30 s; 54 °C, 30 s; 72 °C, 45 s); 72 °C, 420 s	686	F-ACGGCTCTTGAAATGAA R-GCTCTGATACCATCCATG	Kim et al. (2012)
cLET5E4	<i>R3</i>	CAPS/HhaI	94 °C, 120 s; 35 × (94 °C, 20 s; 55 °C, 20 s; 72 °C, 30 s); 72 °C, 300 s	310	F-CCAGGCATGCTCAATTGGACT R-TTCCTGTTGGACTACTTGTGGA	Huang et al. (2004)
GP 185	<i>R3</i>	CAPS/Bst U1	94 °C, 300 s; 35 × (94 °C, 30 s; 55 °C, 30 s; 72 °C, 45 s); 72 °C, 420 s	440	F-CTGGTAATAGTAGAATGATTTCTCGTC R-TTGTTCATGGAGCACTTGC	Huang et al. (2004)
R3 1380	<i>R3</i>	SCAR	94 °C, 180 s; 35 × (94 °C, 35 s; 60 °C, 45 s; 72 °C, 90 s); 72 °C, 180 s	1380	F-GCTCCGACATGTATTGATCTCCC R-GGCAGGCCATTCTAGCTTCTACAG	Sokolova et al. (2010)
Potato Virus Y RYSC3	<i>Ry_{adg}</i>	SCAR	94 °C, 300 s; 35 × (94 °C, 60 s; 55 °C, 30 s; 72 °C, 60 s); 72 °C, 600 s	320	F-ATACACTCATCTAAATTGATGG R-AGGATATACGGCATCTTTCCG	Kasai et al. (2000)
YES 3-3 A	<i>Ry_{sto}</i>	STS	94 °C, 120 s; 10 × (94 °C, 40 s; 61 °C, 40 s; 72 °C, 60 s); 30 × (94 °C, 40 s; 56 °C, 40 s; 72 °C, 60 s); 72 °C, 300 s	341	3F-TAACTCAAGCGGAATAACCC 3A- AATTCACCTGTTACATGCTTCTGTG	Song and Schwarzfischer (2008)
Potato cyst nematode TG 689	<i>H1</i>	SCAR	94 °C, 120 s; 35 × (94 °C, 20 s; 55 °C, 20 s; 72 °C, 30 s); 72 °C, 300 s	141	F-TAAAACCTTGGTTAGCCTAT R-CAATAGAACGTGTTGTTACCAA	Milczarek et al. (2011)
HC	<i>GpaVvrn.QTL</i> (HC.QRL)	AS	94 °C, 300 s; 94 °C, 60 s; 65 °C, 60 s; 72 °C, 60 s; 6 × (94 °C, 30 s; 65 °C decreasing the annealing temp. to 60 °C by 1 °C/cycle, 30 s; 72 °C, 30 s); 30 × (94 °C, 30 s; 60.5 °C, 30 s; 72 °C, 30 s); 72 °C, 300 s	276	F-ACACCACTGTTGATAAAAAACT R-GCTTACTCCCTGCTGAAG	Sattarzadeh et al. (2006)
Gro1-4	<i>Gro1-4</i>	AS	94 °C, 180 s; 35 × (92 °C, 45 s; 58 °C, 45 s; 72 °C, 60 s); 72 °C, 600 s	602	F-TCTTGGAGATACTGATTCTCA R-CGACCTAAATGAAAAGCATCT	Gebhardt et al. (2006)
Gro1-4-1	<i>Gro1-4</i>	STS	94 °C, 600 s; 35 × (94 °C, 30 s; 62 °C, 45 s; 72 °C, 60 s); 72 °C, 600 s	602	F-AAGCCACAACCTACTGGAG R-GATATAGTACGTAATCATGCC	Asano et al. (2012)

^a AS: allele specific; SCAR: sequence characterized amplified region; CAPS: cleaved amplified polymorphic sequence; STS: sequenced tagged site.

was estimated. The genotypes were categorized from susceptible to highly resistant using the following scale:

Lesion area (cm^2)	Grade
Up to 1.0	Highly resistant (HR)
1.1–2.5	Resistant (R)
2.51–6.0	Moderately resistant (MR)
>6.0	Susceptible (S)

2.3.2. Screening for resistance to Potato Virus Y

The plant material was grown under glass house conditions at the Central Potato Research Institute (CPRI), Shimla, during summer (March–June) at an average temperature from 18 to 22 °C. Twenty days after emergence, the plants were mechanically inoculated with the PVY^O strain. The inoculum was prepared from PVY^O infected susceptible potato plants Kufri Pushkar maintained at CPRI. PVY^O infected leaf tissue (1.0 g) was ground in 2.0 ml 0.1 M potassium phosphate buffer containing 0.1 M EDTA, pH 7.0 (PBE). The slurry was passed through a cotton wool plug to obtain the sap which was centrifuged at 16,000 rpm for 5 min. The clear virus extract (CVE) in the supernatant was used for mechanical plant inoculation by spray gun method (Fernanda-Northcote, 1991). The plant material used for screening PVY resistance was potato tuber plants grown in earthen pots. The plants were observed once per week for PVY symptoms development. One month after inoculation, plants were tested by DAS-ELISA (Voller et al., 1976). PVY resistant genotypes were further confirmed for virus culture purity by immuno electron microscopy (IEM). Collodion film-covered copper grids were coated with PVY^O-antisera raised at CPRI (Khurana et al., 1987). The grids were kept at room temperature for 5 min. The grids were floated on micro-drops of CVE, washed with autoclaved distilled water, drained, placed over Parafilm and fixed on a glass

slide. The slide was kept in a humid Petri dish. The grids were incubated at 37 °C for 1 h and then washed with autoclaved distilled water, drained and stained with 3% (v/v) glutaraldehyde. Detection of PVY^O by IEM was carried out by decoration of the trapped virions. Grids with trapped virions were again floated on micro-drops of 1000-fold diluted PVY^O antisera and incubated at 37 °C for 1 h (Garg et al., 1999). Grids were again washed, drained and stained in 2% (w/v) uranyl acetate, and air-dried before examination in a transmission electron microscope (FEI, USA).

2.3.3. Screening for resistance to potato cyst nematodes

The plants were screened under glasshouse conditions at 18–20 °C by the standard root-ball technique (Van Soest et al., 1983) with slight modification in resistance scale. Five tubers of each clone were planted in 10 cm diameter pots in soil containing 100–110 cysts per 100 ccm soil of both PCN species *G. rostochiensis* and *G. pallida* providing 4000–5000 eggs and larvae per test tuber. Cyst inoculum was prepared using PCN host differentials (Kort et al., 1977). After 60–75 days, when the females are visible on the root balls, the number of females on the root balls of various cultures was recorded. The two *Globodera* species are distinguished by the colour of developing females which is white for *G. pallida* and yellow for *G. rostochiensis*. The clones were categorized based on the scheme shown in Table 2.

3. Results and discussion

In total, 126 potato clones were characterized for the presence of genes for resistance to late blight (*R1*, *R2*, *R3a*), PVY (*Ry_{adg}*, *Ry_{sto}*) and potato cyst nematodes (*HC_QRL*, *H1* and *Gro1-4*) using diagnostic molecular markers (Fig. 1, Supplementary material Table S1). The

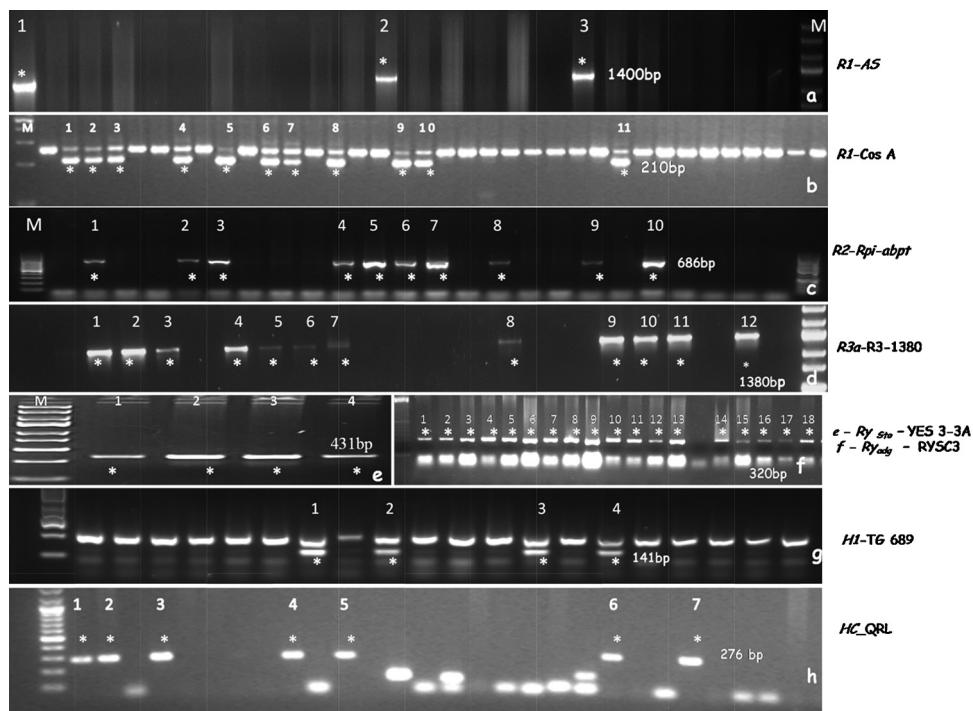


Fig. 1. Markers used to screen the CPRI collection of resistance genes. PCR markers are shown to the right of panels (a) to (h). Marker fragments scored positive are indicated by *. Panels (a) to (d) show markers for major genes for resistance to late blight: *R1* ((a) 1 = CP 4039, 2 = CP 2067, 3 = CP 3771, and (b) 1 = CP 4052, 2 = CP 4056, 3 = CP 4046, 4 = CP 4047, 5 = CP 2137, 6 = K. Khasigarao, 7 = K. Sherpa, 8 = K. Shailja, 9 = K. Kuber, 10 = MP/01-572, 11 = MP/99-322. *R2* ((c) 1 = CP 4052, 2 = CP 4058, 3 = CP 4053, 4 = CP 4050, 5 = CP 4055, 6 = CP 4041, 7 = CP 4044, 8 = CP 4045, 9 = CP 4042, 10 = MP/04-578) and *R3a* ((d) 1 = CP 4052, 2 = CP 4050, 3 = CP 4055, 4 = CP 3400, 5 = MP/2K-424, 6 = B-420, 7 = CP 1945A, 8 = MP/99-322, 9 = HT/93-727, 10 = CP 2067, 11 = CP 3904, 12 = CP 3907. Panels (e) and (f) show markers for extreme resistance to PVY ((e) *Ry_{sto}*: 1 = CP 4042, 2 = LT-1, 3 = CP 1765, 4 = CP 3198 and (f) *Ry_{adg}*: 1 = CP 4054, 2 = CP 4038, 3 = CP 4052, 4 = CP 4056, 5 = CP 4046, 6 = CP 4055, 7 = CP 4047, 8 = CP 4039, 9 = K. Chipsona-1, 10 = CP 3400, 11 = K. Himsiona, 12 = CP 2137, 13 = MP/97-921, 14 = MP/04-578, 15 = CP 3771, 16 = CP 2058, 17 = CP 3904, 18 = CP 3907. Panels (g) and (h) show markers for resistance to *G. rostochiensis* and *G. pallida*, respectively ((g) 1 = CP 4056, 2 = CP 4047, 3 = CP 4039, 4 = K. Neela and (h) 1 = CP 4054, 2 = CP 4052, 3 = CP 4046, 4 = CP 4055, 5 = CP 3400, 6 = CP 2067, 7 = CP 3904). The diagnostic marker fragments size used for scoring are indicated on the right of panel. M is marker size.

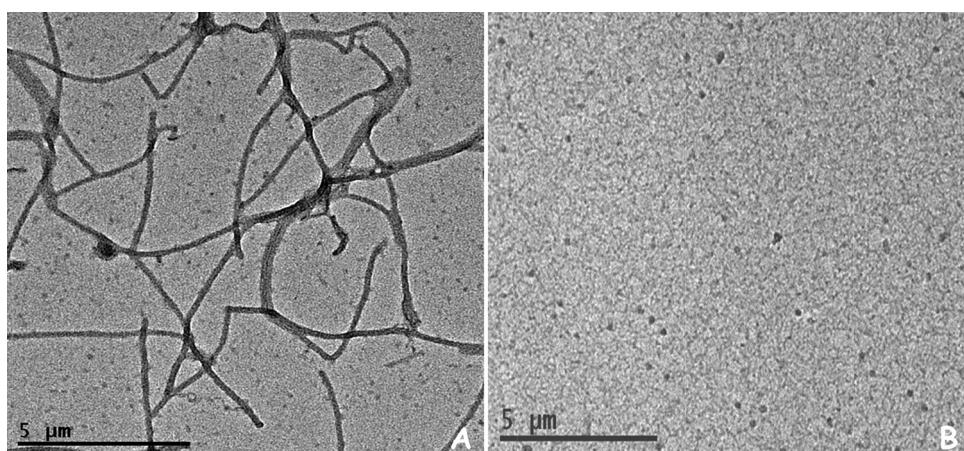


Fig. 2. Presence and absence of PVY particles in susceptible (A) and resistant (B) genotypes, respectively as observed under TEM (transmission electron microscope).

Table 2
Categories for assessing nematode resistance.

Females/root ball	Grade	Resistance
None	0	R
1–5 females	1	R
6–20 females	2	MR
21–50 females	3	S
>50 females	4	S

same clones were phenotypically evaluated for late blight, PVY and nematode resistance.

Supplementary Table 1 related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.scienta.2014.09.018>.

3.1. Late blight resistance screening

Genotyping 22 late blight differentials with markers R1-AS, R1-CosA, R2-Rpi-abpt and R3a-R3-1380 demonstrated marker specificity for R1, R2 and R3 genes (Supplementary material Table S1). Markers R1-AS and CosA gave identical scores. The markers cLET5E4 and GP185 closely linked to the R3 locus were not diagnostic for R3. In concordance with Trognitz and Trognitz (2007) differentials R5, R6 and R9 were also positive for the R1 markers.

Table 3
Mean rank for late blight resistance scores of genotypes with one, two or three R genes in 148 genotypes.

R gene	Number of genotypes	Mean rank
None	57	52.96
R2	18	59.00
R3a	32	84.25
R2, R3a	11	80.14
R1	15	103.83
R1, R3a	5	107.20
R1, R2	5	117.30
R1, R2, R3a	5	137.50

The differentials R5 and R9 scored also positive for R2 and R3a, and differential R6 scored positive for R2. According to the marker scores, the R1 gene was present in 20 genotypes, 28 genotypes possessed R2 and 43 genotypes the R3a gene. Correlation between presence of the marker and phenotypic resistance was tested with a Kruskal-Wallis test. Resistance scores were converted in numerical values 0 (S), 1 (MR) and 2 (R). When using the presence or absence of a single R gene as grouping variable, presence of the R1 gene was highly significantly correlated with resistance (chi-square 35.906, $p < 0.001$), which confirms earlier findings in European germplasm collections (Gebhardt et al., 2004; Sokolova et al., 2010). Presence of

Table 4
The identified promising genotypes (14) with multiple disease resistance genes.

Genotype (s)	Variety name	Source country	Year of introduction/development	Resistance/tolerance									
				Late blight			PVY				PCN		
				Genotypic		Ry _{adg}	Phenotypic	Genotypic	Phenotypic	Genotypic		H1	HC_QRL
				R1	R2					G. pallida	G. rostochiensis		
CP 2067	ASN69-1	Peru	1981	+	—	+	R	—	MR	—	+	MR	S
CP 3771	393371159	Peru	2001	+	—	—	R	+	R	—	—	S	S
CP 4039	391846.5	Peru	2006	+	—	—	MR	+	R	+	—	MR	R
CP 4046	395192.1	Peru	2006	+	—	—	MR	+	R	—	+	R	R
CP 4047	395193.6	Peru	2006	+	—	—	MR	+	R	+	—	MR	R
CP 4052	397029.21	Peru	2006	+	+	+	R	+	R	—	+	MR	MR
CP 4054	397065.28	Peru	2006	—	—	—	S	+	R	—	+	R	R
CP 4055	397069.11	Peru	2006	—	+	+	MR	+	R	—	+	MR	S
CP 4056	397073.7	Peru	2006	+	—	—	MR	+	R	+	—	MR	R
CP 3400	Kufri Jawahar	India	1996	—	—	+	MR	+	R	—	+	MR	S
CP 2137	Kufri Alankar	India	1996	+	—	—	MR	+	R	—	—	MR	MR
CP 3904	MP/97-625	India	1997	—	+	+	S	+	R	—	+	R	R
CP 3907	MP/97-699	India	1997	—	+	+	S	+	R	—	—	MR	MR
MP/04-578	—	India	2004	—	+	—	S	+	R	—	+	R	R

Table 5
Susceptible genotypes lacking resistance genes/markers for late blight, PVY and PCN.

Late blight				PVY				PCN			
Accession/genotype	Variety name	Source country	Year of introduc-tion/development	Accession/genotype	Variety name	Source country	Year of introduc-tion/development	Accession/genotype	Variety name	Source country	Year of introduc-tion/development
CP 3498	R Craig's Royal	Peru	1999	CP 4051	CIP 396244.12	Peru	2006	CP 4058	CIP 397099.6	Peru	2006
CP 2139	Kufri Bahar	India	1980	CP 4058	CIP 397099.6	Peru	2006	CP 4072	Kufri Surya	Peru	2006
CP 2141	Kufri Chandermukhi	India	1968	CP 4053	CIP 397065.2	Peru	2006	CP 4041	CIP 394034.7	Peru	2006
CP 4051	CIP 396244.12	Peru	2006	CP 4057	CIP 397079.6	Peru	2006	CP 4049	CIP 396029.205	Peru	2006
CP 4054	CIP 397065.28	Peru	2006	CP 4050	CIP 396037.215	Peru	2006	CP 4050	CIP 396037.215	Peru	2006
CP 4038	CIP 390637.1	Peru	2006	CP 4041	CIP 394034.7	Peru	2006	CP 2138	Kufri Badshah	India	1979
JEX/A-663	Andigena advance hybrid	India	2000	CP 4044	CIP 395109.29	Peru	2006	CP 3403	Kufri Giriraj	India	1998
JEX/A-695	Andigena advance hybrid	India	2000	CP 4045	CIP 395112.6	Peru	2006	CP 2145	Kufri Khasigar	India	1968
CP 4072	Kufri Surya	India	1999	CP 4043	CIP 395017.229	Peru	2006	CP 3402	Kufri Pukhraj	India	1998
CP 3871	B-420	India	2003	CP 4049	CIP 396029.205	Peru	2006	CP 4105	Kufri Girdhari	India	2008
MP/92-35	Advanced processing hybrid	India	1992	CP 4048	CIP 395195.7	Peru	2006	CP 2156	Kufri Sheetman	India	1968
CP 4057	397079.6	Peru	2006	CP 4049	CIP 396029.205	Peru	2006	CP 4073	Kufri Pushkar	India	1985
CP 2294	P-4	Peru	1987	MP/2K-424	Advanced processing hybrid	India	2000	CP 4099	Kufri Himsona	India	2008
CP 3893	Kufri Khyati	India	2008	MP/99-1189	Advanced processing hybrid	India	1999	CP 2157	Kufri Sherpa	India	1983

R2 or *R3a* was not significantly correlated with resistance. However, stacked *R* genes improved resistance (chi-square 54.513, $p < 0.001$) as shown in Table 3. The mean rank was lowest for genotypes lacking any of the three *R* genes and highest for five genotypes having all three *R* genes. The five genotypes combining all three *R* genes (CP 4052, 3503(R5), 3495 (R9), 3514 (1.2.3), 3516 (1.2.3.4)) all scored resistant. Thus the combination of different resistant genes can have a cumulative effect on horizontal resistance against *P. infestans*.

3.2. Potato Virus Y resistance screening

Of 126 genotypes screened, 18 were positive for the RYSC3 marker. Without exception, these 18 genotypes were highly resistant to PVY (Supplementary material Table S1), confirming the diagnostic value of the RYSC3 marker for the presence of the *Ry_{adg}* gene as corroborated in many previous studies (Boris et al., 2009). All genotypes possessing this gene were negative by ELISA assay and were also highly resistant to PVY under field conditions. Only four genotypes CP4042, LT-1, CP1765 and CP 3198 were positive for the *Ry_{sto}* gene according to marker YES3-3A. All four genotypes were resistant to PVY, showing negative ELISA reactions and no symptoms of PVY infection. Further, electron microscope scans results of PVY particles in these experiments were in concurrence with ELISA results. The genotypes with positive ELISA signal showed the presence of PVY particles as observed under TEM electron microscope (Fig. 2). However, 38 genotypes were resistant to PVY but were negative for both the RYSC3 and YES3-3A marker. Interestingly, no PVY particles were detected in these genotypes under electron microscope. Though, the interaction of signalling molecules like salicylates, jasmonates, ethylene etc. with virus plays important function in infection, multiplication and symptoms development, and so controls the resistance mechanism (Baebler et al., 2011) but their role in resistance genetics is not well documented. The genotypes showing negative PCR marker assay, may possibly be due to new recombination of linking *R* gene with the marker, or due to the presence of one or more additional sources of PVY resistance other than the tested genes viz., *Ry_{adg}* and *Ry_{sto}* in these genotypes. The genotypes can be the basis for future studies aimed at localization of the resistance genes and development of diagnostic markers.

3.3. PCN resistance screening

Screening 126 genotypes with the marker TG689 revealed 12 genotypes positive for the DNA fragment that is diagnostic for the *H1* gene, of which 10 were highly resistant to *G. rostochiensis*, one showed moderate resistance (CP 4043) and one was susceptible (CP 1911). The Mann–Whitney test for an association between the diagnostic DNA fragment and resistance to *G. rostochiensis* was highly significant ($p < 0.001$). This confirms the usefulness of the TG689 marker for diagnosis of the *H1* resistance gene. However, recombination between the diagnostic DNA fragment and the *H1* resistance allele seems occasionally to occur. The *Gro1-4* specific DNA fragment (Gebhardt et al., 2006) was detected in 46 genotypes, but the presence of this fragment was not diagnostic for resistance to *G. rostochiensis* (data not shown). Screening the collection with the improved marker *Gro1-4-1* (Asano et al., 2012) failed to amplify the specific PCR fragment in any genotype (data not shown), probably due to the absence of the *Gro1-4* gene altogether in the material analysed. This information implicates that breeding novel genotypes and varieties having the *Gro1-4* resistance gene from *S. spegazzinii* should broaden the PCN resistance basis in Indian breeding programmes. On the other hand, 12 genotypes negative for the *H1* marker were highly resistant to *G. rostochiensis*.

Some of those might be recombinants that have lost the diagnostic TG689 marker fragment, or alternatively, nematode resistance genes other than *H1* and *Gro1-4* are present in the collection, for example from *S. vernei*. Twenty-five accessions were positive for the HC_QRL marker imparting resistance against *G. pallida* pathotype Pa2/3, among those nine accessions with high resistance to *G. rostochiensis*. The Mann–Whitney test for association between the presence of the HC marker and phenotypic resistance was significant for both resistance to *G. rostochiensis* ($p = 0.006$) as well as *G. pallida* ($p < 0.001$).

Based on molecular markers and phenotyping analyses we identified 14 potato genotypes possessing at least two genes for resistance to late blight, Potato Virus Y and/or cyst nematodes (Table 4). These genotypes included nine germplasm accessions (CP 2067, CP 3771, CP 4039, CP 4046, CP 4047, CP 4052, CP 4054, CP 4055, CP 4056), two commercial Indian potato cultivars (Kufri Jawahar and Kufri Alankar) and three advanced potato hybrids (MP/97-625, MP/97-699 and MP/04-578). Three of the 14 genotypes were resistant and 7 were moderately resistant to late blight. All except one genotype were resistant to PVY due to the presence of the *Ry_{adg}* gene. Four and nine genotypes were resistant and moderately resistant, respectively, to *G. pallida*. Seven and three were resistant and moderately resistant, respectively, to *G. rostochiensis*. Eight elite genotypes possessed a maximum number of four to five genes conferring resistance against multiple diseases. These are clones CP 2067, CP4039, CP 4046, CP 4047, CP 4052, CP 4056, CP 2137 and CP 3904. Genotypes devoid of any markers diagnostic for resistance genes and susceptible for the disease are listed in Table 5. This study validates the association of phenotypic resistance with the presence/absence of diagnostic marker linked with gene for late blight, PVY and cyst nematodes.

4. Conclusions

Potato breeders have always endeavoured to breed varieties with multiple resistances to biotic stresses. However, owing to the polygenic nature of resistances, tetrasomic inheritance in potato and lack of effective screening techniques to screen large segregating progenies, selection of such 'wonder' genotypes remained largely elusive. With the advent of molecular markers, it has become feasible to screen large populations at early stages and make effective selections of parental and progeny genotypes without extensive phenotypic testing. The markers used in this study are either tightly linked to the genes of interest or part of the gene and hence recombination events rarely occur between the marker and gene of interest. As potato is a clonally propagated crop, the genetic architecture once obtained will be fixed in subsequent clonal generations. Therefore, the chances of success are very high when breeders use MAS as an aid in the potato breeding programme. The results of our study are in agreement with this hypothesis. Using molecular markers, we have identified 14 multiple resistant genotypes in the CPRI germplasm collection. The resistant phenotypes were confirmed by established evaluation methods in the laboratory, greenhouse and field. Thus, we were able to identify some elite potato genotypes which can be evaluated straight forward in the hot spots for agronomic superiority and, if found suitable, can be released as new cultivars. Besides that they can be used as parents in various resistance breeding programmes. Our study also identified several resistant cultivars that lack any of the tested major genes for resistance to PVY and PCN. The reason for this could be, besides recombination between *R* gene and marker, the presence of new resistance sources and possibly new types of resistance. It might be worthwhile to contemplate mapping and marker development for such genes.

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