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Genetic multiplex state study of some advanced potato clones conferring *Potato virus Y* ^{NTN} (PVY^{NTN}) extreme resistance

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ABSTRACT

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***Potato virus* Y is known as the most important potato-infecting agent in many parts of the world. In this study, the genetic structure of 16 advanced potato clones that carry the alleles of *Ry* as an extreme resistance gene were investigated. Additionally, multiplex (duplex, triplex or quadruplex) state of genetic of selected clones, where the alleles originate from different species such as *Solanum stoloniferum* (4X), *S. tuberosum* subsp. *andigena* (4X), *S. hougassi* (6X), were evaluated using artificial mechanical and graft inoculations under greenhouse conditions. ELISA was used to confirm experimental materials infection. Results showed that potato clones designated as 96.353, 97.557, 97.559, 97.560, 98.433, 99.373, 99.384, 397097-2, 397082-10, 397074-9, 397081-1 and 396136-1 contain extreme resistance genes (ER) against PVY^{NTN}. These selected clones were crossed with a PVY^{NTN} susceptible line, S440. At least 200 genotypes of each family were evaluated in three replications using mechanical infection with the PVY^{NTN}. *Chi* square test was used to prove the fitness of observed segregation ratios in comparison to predicted ones. The results of X^2 test showed that 99.373, 99.384 and 98.433 clones follow the duplex manner while 96.353, 97.560 and 97.559 were carrying the resistance gene in a simplex state. For 97.557, 397097-2, 397082-10, 397074-9, 397081-1 and 396136-1, there was a significant difference between the observed and expected ratios even for simplex, duplex or triplex stages. The identified duplex genotypes have the potential to provide a durable PVY genetic resistance and can decrease the virus titer in the harboring genotype.**

Keywords: hetero multiplex, potato, resistance

INTRODUCTION

Potato is known as the host of different kinds of pathogens including fungi, bacteria, phytoplasmas, viruses, viroids and nematodes, those cause quantitative and qualitative yield losses. In many cases, viral potato crop infections result in reduced yield and vegetative growth accompanied by symptoms such as leaf rolling, curling, mosaic, inter-veinal banding, leaf drop, plant stunting and production of small or undersized tubers (Jayashige *et al*., 1989; Beukema and van der Zaag, 1990). Due to the vegetative reproduction of potatoes, viral diseases increase in severity over the seasons once the crop is infected, leading to successive yield and crop quality losses. In other words, viral infection of a susceptible potato clone extends to subsequent generations, resulting in seed degeneration. (Hide and Lapwood, 1992; Omer and El-Hassan, 1992; Rahman *et al*., 2010).

As potato viral diseases are vectored non-persistently, they cannot be controlled by pesticide use, making it necessary to produce virus free potato plantlets and apply costly seeds certification protocols. Deployment of resistant cultivars, particularly extreme resistant, is the cost-effective and reliable approach to manage potato-infecting viruses. Achieving this type of resistance is one of the most favorable goals for breeders. Extreme resistance (ER) genes are effective at the single cell level, providing resistance against all strains of the virus and preventing virus multiplication at early infection stages (Ruth *et al.,* 2001). In spite of hypersensitivity reaction (HR) conferring gene, a rapid defence response, they are not influenced by different environmental conditions and plant physiological status (Ahmadvand and Mousapour

Gorji, 2016).

The most important potato viruses are *Potato leaf roll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato virus A* (PVA), *Potato virus S* (PVS) and *Potato virus M* (PVM) (Ahmadvand *et al.*, 2012; Hide and Lapwood, 1992; Evans *et al*., 1992; Strange and Scott, 2005).

Potato leaf roll virus is probably the most damaging and widespread potato virus, while recently the importance of PVY has dramatically increased worldwide due to the appearance of a new tuber necrotic strain, PVY^{NTN}. PVY is aphid-transmitted in a non-persistent manner and cannot be controlled using aphicides (De Bokx and van der Want, 1987). It is typical member of the genus *Potyvirus* (*Potyviridae* family), containing 179 approved and tentative species (Rajamaki *et al.* 2004; Fauquet *et al.* 2005; Shukla *et al*. 1994; ICTV, 2017). PVY^{NTN} is a subgroup of PVY^{N} causing potato tuber necrotic ring spot disease (PTNRD) (Beczner *et al.*, 1984). Infections by PVY^{NTN} can induce a rapid and severe systemic veinal necrosis and results in severely damaged tubers that are not suitable for marketing and storage (Beczner *et al.*, 1984; Szajko *et al.*, 2008). Symptom development following PVY^{NTN} infection depends on many factors, not all of which are yet fully elucidated (Le Romancer *et al*.; 1994, Browning *et al.*, 2004).

Breeding strategies can be designed to develop genotypes having resistance genes against more than one pathogen or pest (multiplex resistance I.), or to develop genotypes where alleles of certain resistance genes can originate from one (Multiplex II/a.) or even from several different wild potato species (Multiplex II/b=Hetero multiplex). The species used in breeding programs as donors of tolerance and resistance traits are particularly *Solanum demissum, S. acaule, S. chacoense, S. spegazinii, S. stoloniferum* and *S. vernei* (Caligari, 1992; Mousapour Gorji *et al.*, 2011; Ahmadvand *et al.*, 2013). *S. stoloniferum* and *S. demissum* are characterized as the main resistance sources against virus and late blight , respectively (Hawkes, 1990).

Hybrids containing large proportions of wild potato genomes may express multiple resistances due to enriched disease resistance genes located in their wild relatives. Jansky and Rouse (2003) identified resistance against several diseases in diploid interspecific hybrids populations of potato. Chen and colleagues (2003) identified multiple resistance in wild solanum species against late blight, Colorado Potato Beetle (*CPB*) and blackleg (*Pectobacterium carotovorum*). Similarly, De Maine and coworkers (1993) argue that *Solanum phureja* group is a valuable source of multiple disease resistance genes. Incorporation of disease resistance genes from different source of wild potatoes could release clones or parental lines carrying resistance genes in multiplex state. Solomon-Blackburn and Barker (1993) created clones with strong PLRV resistance by combining genes that limit virus multiplication resulting in resistance. Colon and colleagues (1995) combined minor resistance genes against late blight originating from four wild *Solanum* species with diploid *tuberosum* group clones. Murphy and coworkers (1999) used conventional hybridization between two tetraploid potato clones, each bearing different disease resistance traits, to create a clone that was resistant against several diseases. Resistance genes frequently encode resistance to some but not entire isolates of a certain pathogen. The Ry gene confers the ER type of resistance against PVY. Ry_{adg}, derived from *Solanum tuberosum* ssp. *andigena*, and Ry_{chc}, from *S. chacoense*, were mapped on chromosomes XI (Hamalainen *et al*., 1997) and IX (Hosaka *et al*., 2001), respectively. Another gene for extreme resistance to PVY, Ry_{sto} from *S. stoloniferum*, has also been mapped on chromosome XII (Flis *et al.*, 2005). To date, Ry genes have not been sequenced, they perhaps belong to the nucleotide-binding site and leucine-rich repeat (NBS-LRR) class of resistance genes (Gururani et al., 2012)

Mendoza *et al*. (1996) created parental lines including the *Ry* gene of *S. tuberosum* ssp. *andigena* in triplex format. Polgár *et al*. (2002) developed duplex breeding lines where alleles of a resistance gene to PVY originated from *S. stoloniferum, S. hugasii* and *S. tuberosum* ssp. *andigena.*

The objective of the current study was to identify the genetic structure of new potato genotypes carrying alleles of *Ry* gene against PVY^{NTN} in a multiplex state (duplex, triplex or quadruplex). These alleles are originating from different species such as *S. stoloniferum* (4X), *S. tuberosum* ssp. *andigena* (4X), *S. hougassi* (6X).

MATERIALS AND METHODS

Genotypes:

Seven (96.353, 97.557, 97.559, 97.560, 98.433, 99.373 and 99.384), and nine (397097-2, 397082-10, 397074-9, 397081-1, 396136-1, 397031-16, 397045-13, 397067-2, and 397081-9), advanced potato clones bred by Potato Research Center (PRC, Hungary), and International Potato Center (CIP), respectively, were used in this study. The pedigree of studied genotypes is illustrated in Table 1.

Experimental potato clones were treated using electro and chemotherapy techniques to eliminate any possible viral infections and then subjected to

Table 1. Pedigree and reaction of experimental clones against PVY^{NTN} based on mechanical and graft inoculation assays using DAS-ELISA.

Clone Accession No	origin	Pedigree	graft inoculation				mechanical inoculation				Final reaction
			Rep 1	Rep 2	Rep 3	Reaction	Rep 1	Rep 2	Rep 3	Reaction	
397097-2	CIP	unknown	R	R	R	R	R	R	R	R	R
397082-10	CIP	unknown	R	R	R	R	R	R	R	R	R
397081-1	CIP	unknown	R	R	R	R	R	R	R	R	R
396136-1	CIP	unknown	R	R	R	R	R	R	R	R	R
397074-9	CIP	unknown	R	R	R	R	R	R	R	R	R
99.373	Hungary	88.175 x 89.451[a]	R	R	R	R	R	R	R	R	R
99.384	Hungary	88.175 x 89.451[b]	R	R	R	R	R	R	R	R	R
98.483	Hungary	90.364 x 89.451[c]	R	R	R	R	R	R	R	R	R
96.353	Hungary	90.350 x 89.451[d]	R	R	R	R	R	R	R	R	R
97.560	Hungary	90.315 x 76.9104/1[e]	R	R	R	R	R	R	R	R	R
97.559	Hungary	90.315 x 76.9104/1[f]	R	R	R	R	R	R	R	R	R
97.557	Hungary	90.315 x Kastia[g]	R	R	R	R	R	R	R	R	R
397045-13	CIP	unknown	S	R	S	S	R	R	R	R	S
397081-9	CIP	unknown	S	R	S	S	R	R	R	R	S
397031-16	CIP	unknown	S	S	S	S	R	R	R	R	S
397067-2	CIP	unknown	S	S	S	S	R	R	R	R	S

Where: a) 88.175 = 79.60 x Chieftain, where 79.60 = *S. tub. S. sto*.; b) 88.175 = 79.60 x Chieftain; c) 90.364 = Bakonyi sarga x 84.2367; d) 90.350 = Bakonyi sarga x 84.2367, Bakonyi sarga = *S. tub. x S. sto*., 89.451 = *S. tub. x S. andigena*.; e) 90.315 = Bakonyi sarga x 84.2367, 76.9104/1 = *S. tub. x S. andigena*; f) 90.315 = Bakonyi sarga x 84.2367, 76.9104/1 = *S. tub. x S. andigena*; g) 90.315 = Bakonyi sarga x 84.2367, Kastia = *S. tub. x S. hougassii*; R: Resistant; S: Susceptible (at least one plant was infected following inoculation) (CIP,2007)

DAS-ELISA detection systems for important potato infecting viruses such as PVA, PVX, PLRV, PVS and PVM (Shambhu *et al*., 2008). Virus free clones were propagated on MS media in a growth chamber and then transferred into an insect proof greenhouse. Healthy mini-tubers were harvested after two months.

Virus source

In order to identify PVY^{NTN} extreme resistance of potato genotypes, the reaction of 16 advanced potato clones to this virus were evaluated under greenhouse conditions (22˚C temperature, 16 h of light per day/40 K Lux, and 70% relative humidity) at SPII and PRC. Viral inoculum was prepared by using, potato samples with mosaic and vein necrosis symptoms on the leaves and necrotic ring spot in the tubers collected from potato fields in Iran. PVY^{NTN} infection of materials was confirmed by using double antibody sandwich ELISA (DAS-ELISA). DAS-ELISA experiment was carried out based on Clark and Adams using commercially polyclonal and monoclonal antibodies (Bioreba AG, Switzerland) were prepared against necrotic strains of PVY in accordance with the manufacturer's instructions. The enzymatic reaction was recorded at 405 nm wavelength using a Biotek-ELX-800 (USA) plate reader. A sample was considered as a positive reaction when its absorbance was at least two fold the average of four negative controls (threshold value) after 60 to 120 min incubation at room temperature. PVY^{NTN} infected potatoes were biologically purified on *Physalis floridana* and tested on *Physalis floridana* (Fig.1). Inoculum was propagated on healthy *Nicotiana tabacum cv.* Samson those were maintained on Murashige and Skoog (MS) media (Murashige and Skoog, 1962).

Evaluation of reactions

Reaction of experimental genotypes to PVY^{NTN} virus was evaluated using mechanical and graft inoculations assays in two distinct experiments (mechanical and graft inoculations) considering three replications under greenhouse conditions, and based on International Potato Center (CIP) protocol. (CIP, 2007).

For mechanical inoculation, four to six leaf stage experimental potato plants were dusted with carborundum powder (600 mesh). Inoculum was prepared using PVY tobacco infected leaves in phosphate buffer (pH 7.5, 0.1 M K_2HPO_4, 0.025 M KH_2PO_4) and mechanically inoculated on experimental potato clones (Whiteworth *et al*., 2009). Inoculated plants were rinsed using tap water five minutes post inoculation. Four weeks after inoculation, the reaction of each inoculated plant was assessed using DAS-ELISA. To identify the type of resistance (extreme or hypersensitivity resistance), the virus free tomato seeds *cv* Rutgers, nucleus seeds, were planted and grown in an insect proof cage. The tomato seedlings were inoculated with PVY^{NTN} and their infection was confirmed by DAS-ELISA. Then, resistant identified genotypes in mechanical inoculation tests were subjected to reciprocal graft inoculation on PVY infected tomato in three replications and were examined by DAS-ELISA after 30 days.

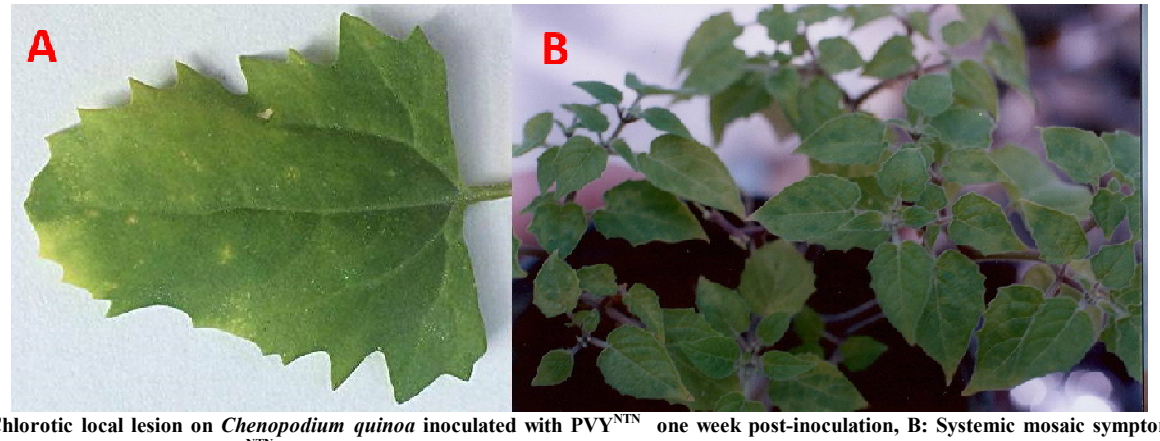

Fig. 1. A: Chlorotic local lesion on *Chenopodium quinoa* inoculated with PVY^{NTN} one week post-inoculation, B: Systemic mosaic symptoms on *Physalis floridana* inoculated with PVY^{NTN} three weeks post-inoculation

Genetic study

Seven and five PRC and SPII clones were carrying *Ry* gene and were extremely resistant against PVY^{NTN} selected from previous experiments (Unpublished data). The germplasm originated from *S. stoloniferum*, *S. hougasii* and. S. *andigena* and were selected based on their pedigree (Table 1) and crossed with a PVY^{NTN} susceptible line, S440. From each cross, the generated true potato seeds (TPS) were planted to create an F_1 population. At

least 200 genotypes of each F1 family were evaluated in three replications, three tubers from each genotype, after mechanical and graft inoculations using purified PVY^{NTN} inoculum. Viral infections were determined by using DAS-ELISA four weeks post inoculations. Twofold higher than healthy plants absorption was considered as a measure indicating infected plants. The copy number of the *Ry* gene was deduced by calculating the ratio of resistant and susceptible plants. *Chi* square test (X^2) at 0.5 and 0.01 probability levels were used to demonstrate the fitness of observed and predicted segregation ratios based on the following basic *chi* square formula. When there is only one degree of freedom, an adjustment known as the Yates correction for continuity must be employed. To use this correction, a value of 0.5 is subtracted from the absolute value (irrespective of algebraic sign) of the numerator contribution of each cell to the above basic computational formula (Yates, 1934).

$$X^2 = \Sigma \frac{(|\text{Observed frequency} - \text{Expected frequency}| - 0.5)^2}{\text{Expected frequency}}$$

RESULTS

Evaluation of reactions showed that studied advanced clones were entirely resistant against PVY^{NTN} based on mechanical inoculation assay. Graft inoculations on PVY^{NTN} infected tomato plants, revealed that clones 397031-16, 397045-13, 397067-2 and 397081-9 were susceptible to the virus (Fig 2a). Regarding resistance reaction in mechanical inoculation, the type of resistance of above clones was assumed as hypersensitive resistance (HR) (Fig 2b). ELISA results showed that the other advanced clones including 96.353, 97.557, 97.559, 97.560, 98.433, 99.373, 99.384, 397097-2, 397082-10, 397074-9, 397081-1, and 396136-1 were resistant based on graft inoculation, as well. Therefore, these genotypes were considered as extreme resistant against PVY^{NTN} and used as parental lines for genetic study (Table 1).

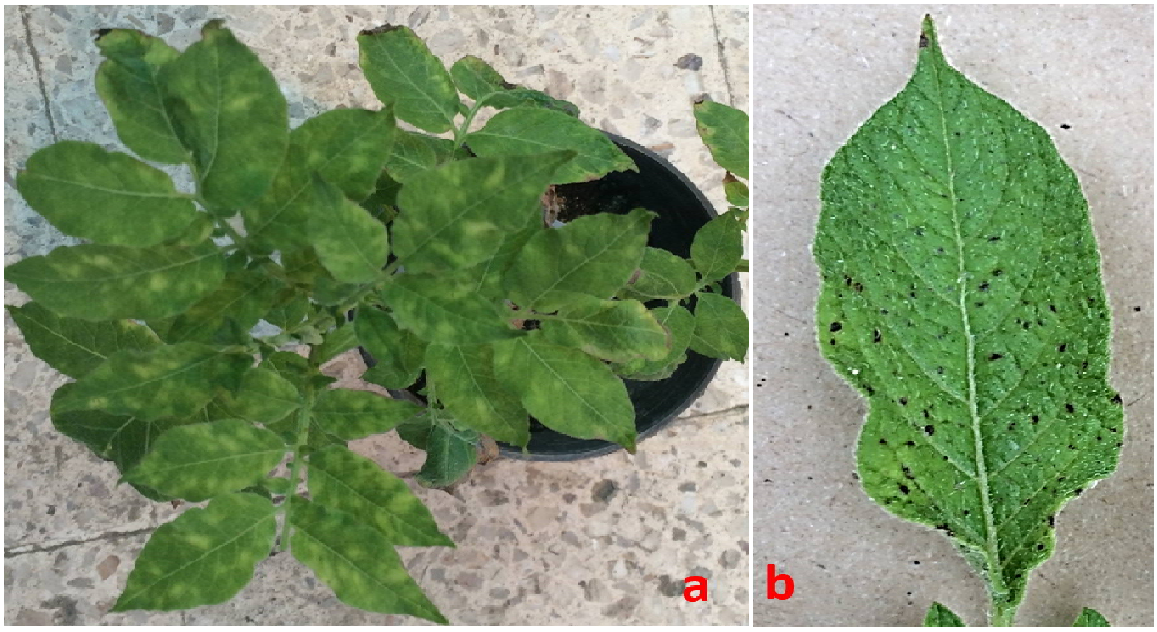

Fig. 2. a: Mosaic symptoms on susceptible potato clones following mechanical inoculation, b: Hypersesitive response to PVY following graft inoculation of potato on infected tomato.

According to the pedigree information (Table 1), the above-mentioned clones should theoretically carry the PVY^{NTN} resistance genes in multiplex state (duplex, triplex or quadruplex). To determine the number of *Ry* genes, the obtained data from ELISA test were analyzed using X^2 test. Results for the 96.353 clone revealed that out of 200 inoculated plants, 117 and 83 plants were non-infected and infected, respectively. The observed ratio for clone 96.353 was similar to expected ratio (Table 2) of simplex manner and it is confirmed by Chi^2 test (Table 3). Results for 97.560 and 97.559 clones were the same as 96.353 clone and Chi^2 supports that they are carrying resistance gene in simplex states (Table 3). The results revealed that genotypes 99.384 (*Ry* gene originated from *S. stoloniferum*. and *S. andigenum*), 98.433 and 99.373 (*S. stoloniferum, S. hougasii,* or S. a*ndigena* (*S.and)*.) are duplexes at 0.05 probability level. We would accept any data set yielding a calculated X^2 value less than 3.84 and 6.64 with a single degree of freedom at 5% and 1% probability levels, respectively. Hence, with regard

to the present study, the calculated X^2 equal to 3.27, 2.17 and 1.24 could be a result of sampling error, mechanical mixing of different germplasm or natural mutation. For 97.557, 397097-2, 397082-10, 397074-9, 397081-1, and 396136-1, there was a significant difference between the observed and expected ratios even for simplex, duplex or triplex states. We assumed that they could be simplex states, however, more confirmations are required.

Table 2. Expected ratios for genotypes and alleles in potato

Genetic bases of parental line	Cross type			Genotype ration of offspring					Allelic ratio	
				AAAA	AAAa	AAaa	Aaaa	aaaa	A	a
Simplex	Aaaa	×	aaaa	0	0	0	18	18	1	1
Duplex	AAaa	×	aaaa	0	0	6	24	6	5	1
Triplex	AAAa	×	aaaa	0	18	18	0	0	1	0

Table 3. Segregation ratio of potato breeding lines for PVY resistance in test crosses

Tested parental lines	Plants tested No. *	Resistant plants No.	Susceptible plants No.	*Chi square* **	Multiplex state
99.373	246	212	34	1.24	Duplex
99.384	241	192	49	2.17	Duplex
98.433	200	157	43	3.27	Duplex
96.353	200	117	83	5.45	Simplex
97.560	200	116	84	4.81	Simplex
97.559	200	109	91	1.45	Simplex
97.557	200	136	64	25.21	Simplex ***
397097-2	200	134	66	22.45	Simplex ***
397082-10	200	135	65	23.80	Simplex ***
397074-9	200	131	69	18.61	Simplex ***
397081-1	200	137	63	26.64	Simplex ***
396136-1	200	136	64	25.21	Simplex ***

*** All available individuals were tested**

****The value of $X^2_{0.05}$ and $X^2_{0.01}$ with single degrees of freedom are equal to 3.84 and 6.63, respectively**

***** Based on mechanical and graft inoculation assays, we assumed that they could be simplex states however, more confirms are required.**

DISCUSSION

From practical point of view, all quadruplex, triplex, duplex and simplex states potato genotypes provide ER against the virus. However, the rate of transmission of ER as a function of the genotype of the resistant progenitor (F_1) is different in each resistant state. Triplex and quadruplex genotypes, called multiplex, have great value in potato breeding programs due to their higher percentage of ER possessing offspring clones (96.4% and 100% ratio, respectively) resulting from crosses with a susceptible parent. Hence, using the breeding lines, they have a resistance gene in quadruplex, triplex or even duplex states, and can increase the rate of resistant genotypes in subsequent progenies. Consequently, the selection process for the combination of resistance along with important agronomic values can be more effective. Various breeding efforts have been undertaken to develop parental lines harboring PVY resistance alleles originating from different wild potato species and conferring in hetero multiplex state. It is considered that this kind of resistance could be more durable against resistance breaking isolates of the virus. Murphy *et al*. (1999) used a conventional hybridization method between two tetraploid breeding clones with different disease resistance traits to create a clone with resistance against several diseases. Solomon-Blackburn and Barker (1993) created clones with strong PLRV resistance through genes combination those reduce the virus titer.. Colon *et al.* (1995) combined minor late-blight resistance genes from four wild solanum species and diploid Tuberosum group clones.

In the present study, we tried to discover the genetic basis of some Hungarian and Iranian advanced potato clones to find parental lines those were potentially carrying *Ry* alleles gene against PVY^{NTN} in duplex and triplex state originating from *S. stoloniferum* (4X), *S. tuberosum* ssp. *andigena* (4X) and *S. hougasii* (6X). As a result, we identified three duplex genotypes where the extreme resistance genes of the two species are combined. The *Ry* resistant gene originated from different wild species and is introgressed into the studied genotypes to enhance the ratio of resistant offspring against PVY^{NTN} during the breeding process.

These advanced lines can effectively be used in breeding programs, directing a combination of PVY^{NTN} resistance with good quality traits of some virus sensitive varieties by increasing the ratio of PVY^{NTN} resistant genotypes in progenies. Moreover, the combination of different sources of *Ry* genes can lead durable resistance against the rather diversified pathogen such as PVY. Spitters and Ward (1988) found that resistance to potato cyst nematodes was more durable in clones with two resistance genes than a single. Polgár *et al*. (2002) developed

different hetero-duplex lines for PVY resistance. In two independent studies, Mendoza and Jayasinghe (1993) and Mendoza *et al.* (1996) developed lines carrying *Ry* gene originating from *S. tuberosum* ssp. *andigena* in the duplex and triplex state.

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