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ARTICLE

Introgression of the RI_{adg} allele of resistance to potato leafroll virus in *Solanum tuberosum* L.

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Abstract: *Genetic resistance to Potato Leafroll Virus (PLRV) is polygenic, which hinders the obtainment of resistant cultivars. However, works carried out at the International Potato Center have identified an andigena accession, LOP-868, with high resistance level and low accumulation of PLRV due to the gene of major effect RI_{adg}. We verify the transfer of the RI_{adg} allele to clones of the cross between LOP-868 and UFLA clones, by using the SCAR RGASC850 molecular marker; to evaluate the reaction of these clones to PLRV by inoculating the virus using aphids; and to analyze their agronomic performance of clones. Among the clones inoculated with viruliferous aphids, 49.3% were negative to the serological test, indicating possible resistance. Clones containing the RI_{adg} allele were identified by the RGASC850 molecular marker, which demonstrates the possibility of transferring the RI_{adg} allele of resistance to PLRV from LOP-868 to Solanum tuberosum. Some clones that presented the RI_{adg} allele are also promising for agronomic performance.*

Key words: *Potato, disease resistance, viral disease.*

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a plant that is vegetatively propagated for commercial purposes, allowing the dissemination of diseases, especially viruses. These pathogens sharply decrease crop yield and quality, known as degeneration (Figueira 1995). In Brazil, viral diseases are more severe than in temperate countries due to the high potential for dissemination by insect vectors, especially the green peach aphid (*Myzus persicae*), since it occurs in every region and season (Gallotti et al. 1992). Despite the possibility of controlling this virus by applying systemic insecticides to reduce the vector population (Lowery and Boiteau 1988), environmental implications and high costs have led to the search for other control strategies, such as genetic resistance, in the context of integrated management.

Potato leafroll virus (PLRV) is considered as one of the main viruses in potato crops in Brazil. It is responsible for more than 50% loss in tuber yield during secondary infection, being historically mentioned as one of the main phytosanitary problems in the production of potato seed in Brazil (Souza Dias and Iamauti 2005).

The obtainment of national cultivars adapted to the Brazilian growing conditions and resistant to the main diseases is the most viable alternative to

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make the crop more yielding and profitable to the producer (Zaag 1987). However, the development of PLRV-resistant cultivars is laborious, since this resistance is governed by several genes, which hinders the selection of resistant genotypes (Cockerham 1945). Another problem relates the interaction of PLRV with other viruses, especially PVX and PVY, which can drastically reduce the level of resistance to PLRV (Brandolini et al. 1992).

However, even with the polygenic control, Velásquez et al. (2007) have demonstrated the existence of a major gene or a genomic region, Rl_{adg}, which controls a single mechanism of resistance to the infection and to PLRV accumulation. The cultivar LOP-868 (*S. tuberosum* ssp. *andigena*) has been mentioned in countless studies as an interesting source of resistance to PLRV, presenting the Rl_{adg} gene in the duplex condition (Mihovilovich et al. 2007, Velásquez et al. 2007, Mihovilovich et al. 2014). Nevertheless, due to the binding of genes that confer resistance to PLRV to undesirable traits, such as irregular shape, small tubers, and low yield (Barker and Harrison 1985), the commercialization of the tubers of this cultivar becomes impracticable.

The objective of this work was to evaluate the reaction of clones obtained from the crosses of a PLRV resistance source (LOP-868), by inoculating them with viruliferous aphids, in order to verify the transfer of the Rl_{adg} allele from the resistance source to *S. tuberosum,* and to detect the presence of this allele by Sequence Characterized Amplified Region (SCAR), and to evaluate the agronomic traits of the experimental clones.

MATERIAL AND METHODS

Trials were carried out at the Plant Virology Laboratory of the Phytopathology Department, at the Laboratory of Molecular Genetics, and in the greenhouse of the Department of Biology, of the Federal University of Lavras (UFLA). In addition, an experiment was carried out in the experimental area of the Agricultural Research Company of Minas Gerais (EPAMIG), in Lambari, MG.

A total of 220 potato clones from six families obtained by biparental crosses between the PLRV-resistant clone LOP-868 and clones from the UFLA Potato Breeding and Genetics Program were evaluated.

Inoculation with viruliferous aphids

In order to evaluate the reaction of the experimental clones, inoculations were carried out using viruliferous aphids (*Myzus persicae*), reared in *Datura stramonium* L. plants in a greenhouse with natural temperature and luminosity. Wingless adults were removed from the leaves and transferred with a fine brush to 15 cm diameter Petri dishes containing leaves of *D. stramonium* L., placed on 1 cm of 1% agar-water. Each Petri dish was sealed with perforated polyvinyl chloride film (PVC), allowing aeration, and was identified and maintained in an air-conditioned chamber at 25 ± 2 ºC, with a relative humidity of 70 ± 10%, with 12-hour photoperiod.

Potato tubers (*S. tuberosum*) contaminated with PLRV, previously evaluated by the *Double Antibody Sandwich - Enzyme Linked Immunono Sorbent Assay* (DAS-ELISA) (Clark and Adams 1977) were planted in 5.0 L pots, and maintained in a greenhouse. Approximately 30 days after seedlings emergence, these plants served as food for healthy aphids, which were placed in contact with these plants for 96 hours for the acquisition access period (AAP). For this, aphids were collected from the leaves of *D. stramonium* L. by using a brush, and transferred to the adaxial part of the leaf of the potato plants. This procedure was carried out under temperature ranging from 20 to 25ºC, and relative humidity between 65 and 90%.

The experimental clones were planted in 0.5 L plastic pots with commercial substrate, and as soon as the seedlings reached between 15 and 20 cm in height, they were inoculated with 50 viruliferous aphids at nymph stage, characterizing a high aphid pressure (CIP 2010). Two plants of each clone were inoculated, characterizing two replications per treatment. The number of replications was ideal for this study; if more plants of each clone had been used, it would have required an increase in the number of aphids, and it would have been more laborious, making the experiment unfeasible. In addition, since the treatments consisted of potato clones, no variation was observed between individuals.

Aphids were placed in contact with each plant for 96 hours for the inoculation access period (IAP), and were collected from the leaves of each inoculum source with the aid of a brush, and transferred to the adaxial part of the leaves of the clones. In order to avoid breaking the stylet of the aphids when they were captured on the leaf, the insects were touched with the brush to stimulate their movement, and were subsequently captured. This procedure was also carried

out in the greenhouse, under cages with anti-aphid screen, with temperature ranging between 20 and 25 °C, and relative humidity between 65 and 90%. After 96 hours, the number of aphids was visualized in the abaxial part of each leaf of the plant, and the total number of aphids per plant was obtained. Afterwards, aphids were eliminated with insecticide.

After two and three weeks aphids had been removed from potato plants, three to four young leaves of each plant were collected in order to perform the DAS-ELISA serological test (Clark and Adams 1977), using polyclonal antisera (Agdia) for PLRV diagnosis.

The cultivars Ágata, Caesar, Markies and Perricholi were used as susceptible control, and LOP-868 was used as resistant control.

Detection of the presence of the Rl_{adg} allele

At approximately 20 to 30 days after planting, young leaves of the plants were collected for DNA extraction by using the method described by Ferreira and Grattapaglia (1998) with modifications.

PCR reaction was performed for a total volume of 25 µL, containing: 5.0 µL 5X Buffer Colorless Go; 1.5 µL $MgCl_2$ (25 mM); 1.0 µL dNTP (10 mM); 0.75 µL of each primer (0.4 µM); 0.125 µL of the enzyme Go Taq Flexi DNA polymerase; 0.25 µL genomic DNA (10 ng); and 15.625 µL ultrapure water. The pair of SCAR primers was used, designated RGASC850, developed by Mihovilovich et al. (2014), which produces an 850 bp fragment, typical of the Rl_{adg} allele.

Amplification reactions were carried out in a thermocycler with the following programming: 35 cycles at 95 °C for 1 minute for denaturation; primer annealing at 46 °C for 1 minute, followed by a final extension for 1 minute at 72 °C. The products obtained from this DNA amplification reaction were then analyzed on 1% agarose gel stained with Gel Red Nucleic Acid Gel Stain.

Cultivar Perricholi was used as susceptible control, and LOP-868 was used as resistant control.

Agronomic evaluation of clones

The field trial was installed in the experimental area of the EPAMIG in Lambari, MG, from October 2013 to January 2014 (lat 21º 58' S, long 45º 22' W, at alt 845 m asl). The region presents high-altitude tropical climate, with annual rainfall of 1642 mm, and average annual temperature of 20.8 ºC. The soil of the experiment is characterized as dystrophic dark red latosol.

An augmented block design with 134 clones was used as regular treatments, distributed in eight blocks with 24 treatments each. Common treatments were the cultivars Cupido, Voyager, Caesar, and clone CBM 16-16. Each plot consisted of a row with five plants spaced 0.35 m x 0.75 m apart.

At planting, fertilization was carried out with the formulation 4-14-8 (N, P_2O_5 e K_2O), at a dose of 3.0 t ha^{-1}, and with soil insecticide (aldicarb) at a dosage of 10.0 kg ha^{-1}. At approximately 40 days after planting, topdressing fertilization was carried out, with 300 kg ha^{-1} ammonium sulphate and 160 kg ha^{-1} potassium chloride, followed by hilling. Phytosanitary treatments were applied throughout the experiment, in order to prevent the competition with weed and the damage caused by pests and diseases, according to the standard of the commercial plantation in the region.

The following agronomic traits were evaluated: i. total tuber yield per plant (g $planta^{-1}$); ii. tubers specific weight, obtained by the formula: SW = air weight (air weight - water weight)$^{-1}$, having the weights determined in a hydrostatic scale; iii. grade of general appearance of tubers, ranging from 1 (poor appearance) to 5 (great appearance), taking into account the shape, skin roughness, and eye depth; the grades were given by three evaluators, being: 1. (round), 2 (round-oval), 3 (oval), 4 (oval-elongated), and 5 (elongated).

Data were subjected to analysis of variance for each trait separately, by using the augmented blocks design (Federer 1956). Analysis of variance was carried out by the mixed procedure of the statistical package SAS (SAS Institute 2008).

RESULTS AND DISCUSSION

Inoculation of PLRV by means of viruliferous aphids

The mean number of aphids plant^{-1} was 77.3. Of the total plants evaluated in the study, 82.3% had mean above 50 aphids plant^{-1}, which can be considered as a high inoculum pressure (CIP 2010).

The results of the serological test for the susceptible controls Ágata, Caesar, Markies and Perricholi were positive, while LOP-868 showed negative results. The cultivars Perricholi and LOP-868 have been used as controls to evaluate resistance to PLRV due to the absence or presence of the Rl_{adg} allele, respectively (Velásquez et al. 2007, Mihovilovich et al. 2014).

Of the experimental clones evaluated in the study, 49.3% presented negative results in the DAS-ELISA serological test at 30 and 40 days after inoculation (DAI), which could be an indication that these clones have resistance level to PLRV. Other works, such as that of Velásquez et al. (2007) and Mihovilovich et al. (2014), who used PLRV inoculation by means of viruliferous aphids, adopted the DAS-ELISA serological test only at 40 DAI, which is enough time for the test to accurately detect the presence or absence of PLRV in the plant.

Beekman (1987) states that resistance to infection is highly dependent on inoculum pressure, which is governed by the population levels of vector aphids. In the present study, of the plants with negative results at 30 and 40 DAI, 77.7% had more than 50 aphids plant^{-1}. On the other hand, of the plants with positive results at 30 and 40 DAI, 81.4% had more than 50 aphid plant^{-1}. In this way, the inoculum pressure was adequate for most treatments, and the application of 50 viruliferous aphids plant^{-1} was ideal for the infection, since under this pressure, 100% of the inoculated susceptible cultivars were infected. According to Mihovilovich et al. (2007), differences between the resistance levels of treatments were defined according to the application of different aphid pressures, and the application of 50 aphids plant^{-1} was considered adequate, since a pressure of aphids per plant higher than this could result in greater damage caused by the pest than by the virus infection.

Identification of the Rl_{adg} allele

The evaluation of the plants by using the RGASC850 marker identified the Rl_{adg} allele. Figure 1 shows two androgenic cultivars, LOP-868 and Perricholi, with PLRV reaction known, and 14 clones with the 850bp band, which identifies the presence of the Rl_{adg} allele. The cultivar LOP-868 is resistant to PLRV and presents the band, whereas the cultivar Perricholi is susceptible and does not present the band, as verified by Mihovilovich et al. (2014).

Of the total number of clones evaluated with the molecular marker, 31% presented the band (presence of the Rl_{adg} allele), and 69% did not present the band (absence of the Rl_{adg} allele). Since the parent LOP-868 presents the Rl_{adg} allele in the duplex condition (Mihovilovich et al. 2014), approximately 83% of the progenies containing the allele were expected.

Of the clones evaluated by both the RGASC850 marker and by the inoculation of PLRV by means of viruliferous aphids, 22.2% presented the band and possible resistance to PLRV at 30 and 40 DAI, i.e., they had a negative result in the DAS-ELISA test; conversely, 29% of the clones did not present the band and had a positive result in the serological

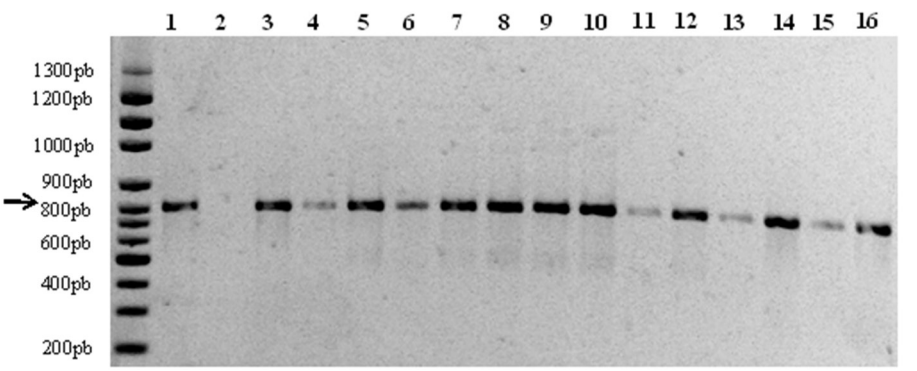

Figure 1. Presence or absence of bands (indicated by the arrow) amplified by the RGASC850 pair of primers by means of PCR, in the resistant [LOP-868 (1)] and susceptible [Perricholi (1)] controls, and in the resistant clones OGC 1-02 (3), OGC 1-12 (4), OGC 2-18 (5), OGC 2-26 (6), OGC 5-07 (7), OGC 5-51 (8), OGC 5-79 (9), OGC 6-44 (10), OGC 6-45 (11), OGC 6-46 (12), OGC 6-52 (13), OGC 6-69 (14), OGC 6-89(15), and OGC 6-105 (16).

test. Both results were expected, since in the presence or absence of the Rl_{adg} allele, the absence or presence of PLRV is expected, respectively.

Approximately 40.3% of the clones did not present the band, nor possible resistance to PLRV at 30 and 40 DAI. These results show that despite the adequate number of aphids per plant, escapes may have occurred, which is a relatively common situation in artificial inoculations, reinforcing the usefulness of the marker for the selection of clones that are resistant to PLRV. In addition, Chuquillanqui and Jones (1980) commented that the technique of transferring aphids from inoculum plants to the treatment with a brush disturbs the aphids, and/or leads to the fatigue of the operator. In the present work, about 4000 aphids were manipulated at each inoculation (totaling approximately 17000 aphids). The acetone technique, developed by Souza Dias et al. (1993), could have avoided operator fatigue; nevertheless, the authors do not confirm that the technique has no effect on the results of the virus transmission.

In addition to escapes, these clones, which are denoted as possible resistant by the inoculation, but do not present the band, can be considered as false positives, due to the recombination of the marker gene with the Rl_{adg} allele. This hypothesis should not be discarded, since even if the marker is at 1.2 cM from the Rl_{adg} resistance allele, a small probability of exchange between the resistance gene and the marker can still be expected. This was the case of some treatments identified as possible false positives in the work of Mihovilovich et al. (2014). Even with the Rl_{adg} allele, PLRV infection can occur and reach levels of up to 20% in LOP 868 when virus pressure is high (Velásquez et al. 2007).

Problems in the detection of PLRV by the DAS-ELISA test may also occur, resulting in false positives or false negatives. In the case of low concentration virus in the plant, as in the case of PLRV (family *Luteoviridae*), inconstant results are described in the literature (Guedes 2000). This fact can be explained by differences in the amount of antigen and the development of each plant. When comparing three techniques to detect *Barley yellow dwarf virus*–PAV-IL (family *Luteoviridae*), Figueira et al. (1997) concluded that the results of the DAS-ELISA test in barley plants were variable, especially when different coatings and/or conjugated antibody preparations were used.

Mihovilovich et al. (2014) also found unexpected results in their study, such as the presence of homomorphic band of the SCAR RGASC850 marker in a highly susceptible cultivar, Flor Blanca. According to these authors, this fact is not surprising, since the potato genome presents high frequency of RGA genomic regions, in addition to homologous sequences (Bakker et al. 2011). Therefore, the presence of homomorphic bands in the clones of the present study also deserves attention, since they were evaluated as possible susceptible clones by the inoculation, and presented the band.

Agronomic performance

Wide variation was observed for the total tuber yield, from clones that produced few grams to clones with production superior to 1000 g $plant^{-1}$. The means of the clones were low; however, approximately 10% of the clone population had a mean above 400 g $plant^{-1}$ (Table 1). The low total tuber yield of the clones is due to the parent LOP-868, which is not adapted and presents several other undesirable traits, such as a great number of small tubers. In addition, the mean temperature throughout the experiment in the field was very high, of approximately 23.10 ºC, hindering the performance of the clones. The ideal temperatures for potato cultivation are between 15 and 21 ºC (Haverkort and Verhagen 2008). The effects of high temperatures have been reported in several works carried out in the south of the state of Minas Gerais (Menezes et al. 2001, Benites and Pinto 2011, Figueiredo et al. 2015). In the present study, the control CBM 16-16 stood out probably due to its tolerance to heat (Menezes et al. 2001).

The specific weight of the tubers is highly correlated with starch content and tubers dry matter, influencing the absorption of oil in the frying process. This trait presented means ranging from 1.0260 to 1.0920. The overall mean of the clones for this trait was low (1.0509), also due to high temperatures. Even the heat-tolerant control CBM 16-16 (Menezes et al. 2001) had a low mean, but it was higher than that of the other treatments (Table 1).

In relation to the shape and appearance of tubers, most of the clones presented round-oval tubers and poor appearance (Table 1). In general, tubers of the clones were small and numerous, which are traits inherited from LOP-868. These traits are probably related to the gene that confers resistance to PLRV (Barker and Harrison 1985). In addition, the high temperatures must have been responsible, in part, for these undesirable traits.

Of the clones tested with the molecular marker RGASC850 and evaluated in the field, 40% presented the Rl_{adg} allele.

Table 1. Adjusted means of the clones with the Rl_{adg} allele, and of the controls for the traits total tuber yield, tuber specific weight, shape grades, and tubers appearance

Clone	**Total yield** (g planta^{-1})	Specific weight	Shape	Appearence
OGC 1-02	174.3	1.0420	1.1	3.1
OGC 1-05	81.8	1.0467	3.3	2.1
OGC 1-10	141.8	1.0443	3.3	1.8
OGC 1-12	314.3	1.0473	1.4	2.3
OGC 1-22	64.3	1.0381	0.7	2.4
OGC 2-01	174.3	1.0414	2.0	2.3
OGC 2-11	36.8	1.0642	2.0	2.8
OGC 2-14	111.8	1.0394	1.8	2.1
OGC 2-16	349.3	1.0551	2.3	2.6
OGC 2-18	216.8	1.0792	2.3	3.0
OGC 2-26	308.1	1.0477	1.4	2.9
OGC 4-02	291.5	1.0508	1.4	2.4
OGC 4-16	253.1	1.0510	2.0	2.2
OGC 5-02	210.2	1.0410	1.3	1.9
OGC 5-07	332.5	1.0589	1.6	3.1
OGC 5-11	95.0	1.0478	3.1	2.6
OGC 5-22	136.4	1.0554	2.0	2.7
OGC 5-37	336.4	1.0487	2.0	2.4
OGC 5-46	182.5	1.0780	1.1	1.9
OGC 5-51	82.5	1.0426	2.1	2.4
OGC 5-58	141.5	1.0455	2.4	1.9
OGC 5-69	121.5	1.0372	2.7	2.9
OGC 5-72	291.5	1.0521	2.7	2.9
OGC 5-75	52.5	1.0767	2.1	2.3
OGC 5-79	114.4	1.0304	2.3	3.1
OGC 6-13	448.7	1.0676	1.9	2.8
OGC 6-16	82.5	1.0916	1.7	2.1
OGC 6-20	261.5	1.0389	2.4	3.1
OGC 6-24	307.5	1.0494	2.1	1.9
OGC 6-33	151.6	1.0654	1.3	2.6
OGC 6-34	289.4	1.0432	1.8	3.1
OGC 6-43	216.4	1.0427	2.0	1.9
OGC 6-44	351.5	1.0422	2.1	3.1
OGC 6-45	495.3	1.0611	2.0	3.1
OGC 6-46	270.3	1.0588	3.5	3.2
OGC 6-52	145.0	1.0673	1.1	2.6
OGC 6-68	332.5	1.0503	2.1	3.6
OGC 6-69	270.3	1.0551	1.7	3.2
OGC 6-82	185.2	1.0367	2.3	2.2
OGC 6-89	315.3	1.0430	3.7	2.6
OGC 6-104	285.2	1.0265	2.3	3.4
OGC 6-105	161.2	1.0407	2.9	2.8
OGC 6-107	252.7	1.0406	2.4	2.9
OGC 6-119	165.8	1.0689	2.1	2.6
General mean of clones	217.1	1.0509	1.9	2.6
CBM 16-16	724.7	1.0592	3.2	3.1
Caesar	359.8	1.0560	3.7	3.1
Cupido	330.8	1.0478	2.9	2.8
Voyager	462.0	1.0525	3.1	3.1
CV (%)	67.8	0.68	32.5	18.1
LSD (5%)[a]	432.45	0.0172	1.70	1.18

[a] Minimum significant difference at 5% probability for means comparison between a control and a clone.

Some clones are worth mentioning, such as clone OGC 6-45, which presented total tuber yield similar to that of the cultivars; tuber specific weight higher than that of the heat tolerant control (CBM 16-16); and a reasonable general appearance of tubers. The clones OGC 2-18, OGC 6-68, OGC 6-34, and OGC 6-44 should also be mentioned (Table 1).

Since some of the parents of the experimental clones also have the Ry_{adg} alleles and/or the Rx_1 allele, which confer extreme resistance to Potato virus Y (PVY) and Potato virus X (PVX), respectively, further trials should be performed on these clones in order to verify the presence of these alleles, adding multiple resistance to these viral diseases.

The inoculation of clones by means of viruliferous aphids with PLRV is laborious and time-consuming, and is not effective in proving resistance or susceptibility of the clones. In order to confirm the resistance or susceptibility, several replications are required. Nevertheless, the association the RGASC850 marker with the inoculation of the clones with aphids leads to greater safety in proving resistance or susceptibility of the clones.

Clones containing the Rl_{adg} allele were identified by means of the molecular marker SCAR RGASC850, showing that the PLRV-resistant Rl_{adg} allele can be transferred from LOP-868 to *Solanum tuberosum*. These clones should be used in backcross programs with commercial potato clones.

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