Resistance to *Papaya ringspot virus*-Watermelon Strain (PRSV-W) in the Desert Watermelon *Citrullus colocynthis*

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Abstract. The bitter desert watermelon, *Citrullus colocynthis* (L.) Schrad is a wild species valuable for biotic and abiotic stress resistance that could be exploited for improving watermelon cultivars [*Citrullus lanatus* (Thunberg) Matsum & Nakai var. *lanatus*]. The objective of this study was to survey and identify *C. colocynthis* accessions displaying resistance to the *Papaya ringspot virus*-watermelon strain (PRSV-W). Thirty-one accessions of *C. colocynthis*, collected in Africa, the Middle East, southwest Asia, and India were evaluated for PRSV-W resistance. Of these 31 accessions, 4 U.S. Plant Introduction (PI) accessions, including 525080 (collected in Qena, Egypt) and PI 537277, PI 652554, and Griffin 14201 (collected at the northern Indian desert of Rajasthan and the neighboring region of Punjab, Pakistan) showed high resistance to PRSV-W. Plants of these four resistant PIs were self-pollinated to produce S1 and S2 seeds that continued to maintain the high levels of PRSV resistance. Since there is a wide genetic distance between watermelon cultivars and *C. colocynthis*, we performed crosses and backcrosses with watermelon cultivars, including ‘Charleston Gray’ and ‘Sugar Baby’ to produce viable seed that would be useful in the development of genetic populations and in introducing the resistance into watermelon cultivars.

PRSV-W, previously known as watermelon mosaic virus-1 (WMV-1), is an important potyvirus causing significant economic damage to cucurbit crops (Bateson et al., 2002). Occurrence of PRSV-W in cucurbit fields coincides with increased aphid populations, particularly in warm climate regions where aphids are present throughout the year (de Azevedo et al., 2012). The PRSV-W has also been frequently present in plants infected with the whitefly-transmitted *Squash*

vein yellowing virus that inflict sudden vine decline in watermelons (Adkins et al., 2008). Insecticide application combined with the use of light-reflective mulches may be a useful strategy to decrease aphid populations and control PRSV-W infectivity in commercial cucurbit fields. However, developing accessions with high resistance to potyviruses is a primary control strategy with significant economic and environmental benefits (Fraser, 1992; Guner and Wehner, 2008). Resistance to PRSV-W was identified and genetic inheritance mode for this important potyvirus was determined in various cucurbit crops (de Azevedo et al., 2012).

Watermelon is an important crop, accounting for 2% of the world area devoted to vegetable production (FAO, Food and Agricultural Organization of the United Nations, 2013). There is a continuous need to identify sources of resistance, useful for enhancing potyvirus resistance in elite watermelon cultivars. Several studies were

conducted to identify watermelon germplasm with resistance to PRSV-W, including studies by Araújo and Souza (1988) and Nascimento et al. (2011), which identified the watermelon accession ‘Ouricuri’ and U.S. PI 595201 as resistant to PRSV-W, respectively. Strange et al. (2002) and Guner (2004) performed extensive screening of U.S. Department of Agriculture watermelon germplasm collection and reported PRSV-W resistance in several PIs of *C. lanatus* ssp. *citroides* collected in southern Africa (PI 244017, PI 244018, PI 244019, PI 482342, PI 482318, PI 482379, and PI 485583) and in *C. lanatus* ssp. *lanatus* PI 595203 collected in Nigeria.

Hojo et al. (1991a, 1991b) identified an African bitter fruit watermelon accession BT-8501 as tolerant to PRSV-W. Still, no sufficient information has been gathered on PRSV resistance in PIs representing the perennial bitter watermelon (*C. colocynthis*) that exists in the deserts of northern Africa, the Middle East, southwest Asia, and India. In a previous study, PIs of *C. colocynthis* exhibited wide genetic diversity compared with *C. lanatus* PIs (Levi et al., 2001), while a recent study indicated that *C. colocynthis* PIs are overall more closely related to watermelon cultivars (*Citrullus lanatus* ssp. *lanatus*) than to PIs of the *C. lanatus* ssp. *citroides* collection (Levi et al., 2013). In this study, we evaluated 31 *C. colocynthis* accessions with the objective of identifying potential source(s) for enhancing PRSV-W resistance in watermelon cultivars.

Material and Methods

Germplasm. All *C. colocynthis* PIs were obtained from the U.S. Department of Agriculture, Agricultural Research Service (USDA, ARS) Southern Regional Plant Genetic Resources Conservation Unit, Griffin, GA. Seven *C. colocynthis* accessions collected in the Negev Desert, Israel (Table 1), were obtained from the Israeli Plant Gene Bank Collection, Agricultural Research Organization (ARO), Volcani Center, Israel. Watermelon cultivars that were used as PRSV-W-susceptible controls and PIs that were used as PRSV-W-resistant controls (PI 595203, PI 244217, and PI 244219) were from selected stock seeds kept at the USDA, ARS, U.S. Vegetable Laboratory (USVL), or at Sakata Seeds, Fort Meyers, FL. All plants were started from seed in standard 50-cell black plastic trays and transplanted into 10-cm nursery pots using a professional commercial potting mix consisting of sphagnum peatmoss, pine bark, sand, and lime. Plants were maintained in an aphid-proof greenhouse. Plants were fertilized biweekly with 150 mg·L⁻¹ Peters Professional 20–20–20 N–P–K (Scotts-Sierra Horticultural Products Company, Marysville, OH).

Experimental design. An experiment was performed with the 31 *C. colocynthis* PIs and with control plants (Table 1) in a greenhouse at the USVL during the winter of 2013, and the experiment was repeated with all 31 PIs

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Table 1. *Citrullus* ssp. accessions, including 31 *Citrullus colocynthis* (CC), 5 *Citrullus lanatus* var. *citroides* (CLC), and 2 heirloom cultivars and a Plant Introduction (PI) representing *Citrullus lanatus* var. *lanatus* (CLL) in this study. The geographic location in which each of the accessions was collected, total number of plants used from both tests (Winters 2013 and 2014), their mean symptom severity (based on total number of plants from both tests), and average enzyme-linked immunosorbent assay (ELISA) values (based on two to four replications) in response to inoculation with *Papaya ringspot virus* (PRSV) in a greenhouse tests at the U.S. Vegetable Laboratory, Charleston, SC, during the Winters 2013 and 2014. Also, negative control plants (not inoculated) and an ELISA buffer are included in this study (on bottom, below separation line). Their overall level of resistance/susceptibility (R = resistant; S = susceptible; IMR = plants shows mild disease reaction) based on their disease severity rating (as described at the material and method section). ELISA tests were performed for all four accessions showing PRSV resistance, and to most accessions showing high susceptibility to PRSV.

Accession	<i>Citrullus</i> ssp.	Geographic location	Number of plants	Phenotype scoring	ELISA	Overall resistance
Griffin 14201	CC	Rajasthan, India	9	1.5 (0.01) ^w	0.1 (0.06)	R
PI 195927		Harage, Ethiopia	8	3.8 (0.1)	0.56 (0.25)	S
PI 220778		Farah, Afghanistan	7	3.3 (0.1)	—	S
PI 346082		Helmand, Afghanistan	8	3.0 (0.95)	—	S
PI 374216		Afghanistan	7	3.2 (0.6)	0.28 (0.13)	S
PI 386014		Iran	7	3.2 (0.15)	—	S
PI 386015		Iran	8	3.0 (0.81)	0.29 (0.17)	S
PI 386016		Iran	7	3.1 (0.31)	—	S
PI 386018		Iran	7	3.2 (0.25)	0.37 (0.16)	S
PI 386019		Iran	7	3.4 (0.3)	—	S
PI 386021		Iran	8	3.2 (1.1)	—	S
PI 386024		Iran	7	3.1 (0.01)	0.32 (0.14)	S
PI 386026		Iran	7	2.9 (0.8)	0.34 (0.11)	S
PI 388770		Morocco	7	3.2 (0.5)	—	S
PI 432337		Cyprus	6	3.9 (0.01)	—	S
PI 525080		Qena, Egypt	7	1.4 (0.01)	0.1 (0.05)	R
PI 525882		Mali	6	3.0 (1.0)	0.28 (0.21)	S
PI 537277		Punjab, Pakistan	9	1.4 (0.01)	0.065 (0.04)	R
PI 537300		Ahal, Turkmenistan	7	3.3 (0.3)	—	S
PI 542616		Algeria	6	3.5 (0.2)	0.8 (0.4)	S
PI 549161		Chad	7	3.9 (1.1)	1.3 (0.8)	S
PI 652554		Rajasthan, India	8	1.5 (0.06)	0.027 (0.06)	R
ARO 13247		Jordan	7	2.0 (1.7)	0.038 (0.01)	S
ARO 18917		Negev Desert, Israel	6	3.6 (0.29)	0.224 (0.09)	S
ARO 18920		Negev Desert, Israel	7	1.7 (0.49)	0.101 (0.01)	IMR
ARO 20587		Negev Desert, Israel	6	2.0 (0.5)	0.449 (0.12)	S
ARO 21031		Negev Desert, Israel	6	3.1 (0.3)	—	S
ARO 22357		Negev Desert, Israel	6	3.25 (0.07)	0.115 (0.12)	S
ARO 22359		Negev Desert, Israel	7	2.8 (0.1)	0.02 (0.01)	S
ARO 23701		Negev Desert, Israel	6	2.9 (0.3)	—	S
ARO 23967		Negev Desert, Israel	6	2.9 (0.31)	—	S
PI 244017	CLC	Transvaal, South Africa	6	2.4 (0.5)	0.38 (0.29)	IMR
PI 244019		Transvaal, South Africa	7	1.5 (0.01)	0.13 (0.037)	R
PI 482246		Zimbabwe	7	3.5 (1.0)	0.82 (0.36)	S
PI 482252		Zimbabwe	6	4.0 (0.3)	1.38 (0.6)	S
PI 482337	CLL	Zimbabwe	7	3.8 (0.3)	—	S
PI 595203		Nigeria	7	2.2 (0.5)	0.08 (0.05)	IMR
Charleston Gray		United States	8	4.3 (0.15)	0.75 (0.31)	S
Long Crimson		United States	12	4.2 (0.1)	1.24 (0.31)	S
² Charleston Gray	CLL	United States	8	1.5 (0.1)	0.06 ^v	
² Long Crimson		United States	6	1.5 (0.1)	0.1	
³ Charleston Gray		United States	4	1.5 (0.0)	0.06	
³ PI 595203		Nigeria	4	1.3 (0.0)	0.12	
³ PI 244019	CLC	South Africa	4	1.3 (0.0)	0.18	
³ PI 482246		Zimbabwe	4	1.5 (0.0)	0.18	
³ Buffer					0.007	

ARO = Agricultural Research Organization.

²Negative control plants (not inoculated) and randomly placed among the PRSV-inoculated plants.

³Plants are grown on a bench in the greenhouse and used as negative control for ELISA test.

^vELISA buffer with no virus.

^wStandard deviation.

^vELISA was performed with one or two plants representing each of the negative control accessions (plants are presumed to be free of virus).

during the winter of 2014. Greenhouse temperatures ranged from 25 to 27 °C (day with 10–12 h of natural lighting period) and 20 to 22 °C (night). In each experiment, three to five plants of each PI were tested and arranged in a randomized fashion among plants of all PIs (Table 1). In addition to the accessions being evaluated in each of the tests (in Winter 2013 and in Winter 2014), there were three to eight positive control plants of the susceptible heirloom cultivars Charleston Gray and Long Crimson as well as three to five negative control plants of each of PIs 595203, 244017,

and 244019 that were previously reported to be PRSV-W resistant (Guner 2004; Strange et al., 2002). All control plants were inoculated with the virus and their positions were randomized among other plants in the experiment. Additionally in each test, two to three plants of the cultivars Charleston Gray and Long Crimson were not inoculated and kept separately in the greenhouse (Table 1). The inoculated checks served as verification of viral infection and the uninoculated checks served as an indicator of other disease in the greenhouse that may confound symptom expression.

Inoculation. The PRSV-W isolate, derived from strain 2052 described by Baker et al. (1991), was obtained from Sakata Seeds, Fort Meyers, FL, and maintained on squash plants (*Cucurbita pepo* L.) in the USVL virology greenhouse. Virus inoculum was prepared by macerating virus-infected squash leaves (1:5 w/v) in 0.01 M phosphate-buffered saline, pH 7.4, with a mortar and pestle (as described by Ling et al., 2009). The inoculation was carried out by first dusting the leaves and cotyledons of young seedlings (three to five leaf stage) with an 800-mesh

carborundum followed by mechanical rubbing with a cotton swab soaked in the virus inoculum (involving several circular motions until the entire leaf was covered). The inoculated seedlings were placed in the shade for 24 h to minimize direct sunlight damage to the newly inoculated leaves. All plants were reinoculated 1 week later to ensure thorough inoculation and to reduce the possibility of escapes (as described by Guner et al. 2002 and Ling et al., 2009).

Symptom severity rating. Three and 4 weeks after the first inoculation, plants were evaluated for virus symptoms, taking into

account the growth habits and leaf morphologies of the different accessions. The rating system used healthy uninfected plants of watermelon cultivars and *C. lanatus* PIs (Table 1) as reference control to identify sick plants resulting from virus infection. Disease severity was rated as: 1—no symptoms; 2—slight mosaic on leaves; 3—mosaic patches and/or necrotic spots on leaves or leaves near apical meristem appearing slightly deformed, yellow in color, or having reduced leaf size; 4—extensive mosaic appearance and severe leaf deformation (as shown in Fig. 1) and reduced growth; 5—extensive mosaic

appearance and severe leaf deformation and the plant is entirely stunted or dead (Harris et al., 2009; Ling et al., 2009).

Enzyme-linked immunosorbent assay. Double-antibody sandwich ELISA tests were performed according to the manufacturer's instructions (Agdia Inc., Elkhart, IN). For the ELISA test the second leaf under the apical meristem was sampled on third week (21 d) after the first inoculation. Leaf extracts were prepared by processing the collected leaf tissue with a homogenizer (Homax-6; Bioreba AG, Reinach, Switzerland) in tissue extraction buffer (1:20 w/v). Microtiter plates were first coated with $1\ \mu\text{g}\cdot\text{mL}^{-1}$ of PRSV antibody, and virus particles were trapped after incubating the tissue extract on the coated plates. Then, the alkaline-phosphatase-PRSV-conjugated antibody was added to the plate. A yellow color developed in positive samples due to enzyme-substrate hydrolysis and was quantified using spectrophotometry with results processed using SoftMax Pro (Molecular Devices, Sunnyvale, CA). A sample with an absorbance value of at least twice that of the mean of the healthy controls (optical density at 405 nm) was regarded as positive (Ling et al., 2009).

Statistical analyses for calculating regression value between PRSV phenotyping and ELISA scores. Bivariate fit of phenotypic scoring by ELISA values was performed to calculate the regression value using the statistical software JMP®, Version 10.0.2 (SAS Institute Inc., Cary, NC, 1989–2007).

Selection of S1- and S2-resistant plants. Plants of most resistant PIs that did not show any virus symptoms (rated 1.0–1.5 on the scale described above) and had ELISA values comparable to those of the healthy plants (Table 1) were selected and self-pollinated in the greenhouse.

Fig. 1. Leaves of the *Papaya ringspot virus* (PRSV)-resistant *Citrullus colocynthis* Plant Introduction (PI) 537277 (upper) vs. distorted leaves of the susceptible *C. colocynthis* PI 549161 (lower) following inoculation with PRSV-watermelon strain isolate, derived from strain 2052 (Baker et al., 1991). The photographs taken 3 weeks after plants were inoculated.

Fig. 2. Bivariate fit of enzyme-linked immunosorbent assay by phenotype scoring using polynomial (quadratic) fit degree, which is statistically significant (P value of 0.05) with an r^2 value of 0.683.

Results and Discussion

Four *C. colocynthis* PIs (PI 537277, PI 585080, PI 652554, and Grif. 14201) did not show any viral symptoms during the 8 weeks following inoculation with PRSV-W. Furthermore, in a third experiment, S1 and S2 generation plants derived from self-pollinating plants of each of these four resistant PIs also displayed resistance to PRSV-W and did not show any viral infection symptoms (corresponding to the data in Table 1, with resistant levels of 1.4, 1.3, 1.4, and 1.4 for PI 537277, PI 585080, PI 652554, and Grif. 14201, respectively) (Fig. 1). The resistance level in these four PIs is comparable to the resistance level in the control PI 244019 and higher than the control PI 595203 or PI 244017, which were previously reported as PRSV resistant (Guner, 2004; Strange et al., 2002). PI 595203 and PI 244017 showed slight mosaic pattern in early stage leaves, but virus symptoms were less noticeable in older leaves (Table 1). Similarly, plants of the *C. colocynthis* accession ARO 18920 (collected at the Negev Desert, Israel) (Table 1) showed some mosaic patterns and slight leaf distortion, but

the symptoms diminished in a later growth. This occurrence implies that a defense mechanism (Freeman and Beattie, 2008) could be involved in attenuating virus symptoms in these accessions.

The ELISA results in this study were consistent with the phenotypic data in PRSV-resistant and susceptible PIs, and in the positive and negative control accessions (Table 1), resulting in a significant regression coefficient (Fig. 2). Most of the *C. colocynthis* accessions in this study were susceptible to PRSV and rated at the range of 3.0 to 4.0 (Table 1), with mosaic, deformed, and/or reduced leaves (Fig. 1); most accessions survived and had slight less symptoms at the sixth week postinoculation. Conversely, several of the infected 'Charleston Gray' and 'Long Crimson' control plants did not survive.

The fact that three of the four PIs identified here as PRSV-W resistant (PI 537277, PI 652554, and Grif. 14201) were collected in the same geographic region (collected at the northern Indian desert of Rajasthan and the neighboring region of Punjab, Pakistan; Table 1) may imply that these three resistant PIs share similar ancestors and the resistance is conferred by the same gene. Strange et al. (2002) and Guner (2004) conducted an inheritance study and allelism test and determined that a recessive gene *prv* confers resistance to PRSV-W in all three *C. lanatus* var. *citroides* accessions (PI 244017, PI 244019, and PI 485583). Further studies are needed to determine the inheritance mode and identify gene loci associated with PRSV-W resistance in *C. colocynthis* PIs.

PI 537277 was also reported to be resistant to *Zucchini yellow mosaic virus* (ZYMV) by Guner (2004). Resistance to potyviruses, including ZYMV and WMV, was identified in watermelon germplasm by Boyhan et al. (1992) and Gillaspie and Wright (1993), respectively. In these studies, several of the *C. lanatus* ssp. *citroides* (PI 482299 and PI 482261) and *C. lanatus* ssp. *lanatus* (PI 595203 and PI 255137) accessions that were resistant to ZYMV also had some resistance to PRSV-W (Guner, 2004). Provvidenti and Gonsalves (1982) found that WMV-resistant *Cucumis metuliferus* accessions were also resistant to PRSV. In a previous study (Ling and Levi, 2007), we identified several *Lagenaria siceraria* PIs collected in India as resistant to both PRSV-W and ZYMV. These results indicate that resistance to these potyviruses might be controlled by common gene(s).

Because of the wide genetic distance between *C. colocynthis* and *C. lanatus* var. *lanatus* (Levi et al., 2001, 2013), the question that is being asked is if the resistant PIs identified here could be a useful source for enhancing PRSV-W resistance in watermelon cultivars (*C. lanatus* var. *lanatus*). In a previous study, we performed crosses and were able to replace the chloroplast and mitochondrial genomes of watermelon cultivars with that of *C. colocynthis* (Levi et al., 2006, 2011). Each of these four resistant

C. colocynthis PIs was crossed with the watermelon cultivars Charleston Gray and/or Sugar Baby by careful hand pollination and produced viable F₁ seeds and plants with reduced fertility (unpublished data). The F₁ plants were backcrossed with same watermelon cultivars to produce BC₁ seeds that will be further used in our attempts to introduce PRSV-W resistance gene loci into the genome background of watermelon cultivars. Also, we have been constructing genetic populations (F₁, F₂, and BC₁R, BC₁S) derived from a cross between PRSV-W-resistant and susceptible *C. colocynthis* lines. These genetic populations will be useful for genetic inheritance and mapping studies and for identification of quantitative gene loci associated with PRSV-W resistance. In the future, using validated markers combined with phenotyping and ELISA, we will be able to incorporate the resistance traits into the genomic background of watermelon cultivars, as has been shown in our recent study with ZYMV (Levi and Ling, 2015).

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