Molecular Breeding

Distorted segregation in Cucurbita pepo populations hampers ZYMV-resistance breeding and candidate gene identification

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Abstract:	Cucurbit crops are economically important worldwide. One of the most serious threats to cucurbit production is Zucchini yellow mosaic virus (ZYMV). Several resistant accessions were identified in Cucurbita moschata and their resistance was introgressed into Cucurbita pepo. However, the mode of inheritance of ZYMV resistance in C. pepo presents a great challenge to attempts at introgressing resistance into elite germplasm. The main goal of this work was to analyze the inheritance of ZYMV resistance and to identify markers associated with genes conferring resistance. An Illumina GoldenGate assay allowed us to assess polymorphism among nine squash genotypes and to discover six SNPs putatively associated with ZYMV resistance/susceptibility. Two F2 and three BC1 populations obtained from crossing the ZYMV-resistant Accession 381e with two susceptible ones, the zucchini 'True French' and the cocozelle 'San Pasquale', were assayed for ZYMV

	resistance. Molecular analysis suggested a close relationship between SNP1 and resistance, which was confirmed using High Resolution Melt (HRM) and a CAPS marker. Distorted segregation in populations segregating for resistance was observed for two SNPs putatively associated with two other genes necessary for expression of resistance. A functional prediction of proteins involved in the resistance mechanisms was performed on genome scaffolds containing the three SNPs of interest. Indeed, 16 full-length Pathogen Recognition Genes (PRGs) were identified around the three SNP markers. In particular, we discovered that two nucleotide-binding site leucine-rich repeat (NBS-LRR) protein-encoding genes were associated closely with the SNP1 marker. The investigation of ZYMV resistance in squash populations and the genomic analysis performed in this work could be useful for better directing the introgression of disease resistance into elite C. pepo germplasm.
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Distorted segregation in *Cucurbita pepo* populations hampers ZYMV-resistance breeding and candidate gene identification

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ABSTRACT

Cucurbit crops are economically important worldwide. One of the most serious threats to cucurbit production is *Zucchini yellow mosaic virus* (ZYMV). Several resistant accessions were identified in *Cucurbita moschata* and their resistance was introgressed into *Cucurbita pepo*. However, the mode of inheritance of ZYMV resistance in *C. pepo* presents a great challenge to attempts at introgressing resistance into elite germplasm. The main goal of this work was to analyze the inheritance of ZYMV resistance and to identify markers associated with genes conferring resistance. An Illumina GoldenGate assay allowed us to assess polymorphism among nine squash genotypes and to discover six SNPs putatively associated with ZYMV resistance/susceptibility. Two F_2 and three BC_1 populations obtained from crossing the ZYMV-resistant Accession 381e with two susceptible ones, the zucchini 'True French' and the cocozelle 'San Pasquale', were assayed for ZYMV resistance. Molecular analysis suggested a close relationship between SNP1 and resistance, which was confirmed using High Resolution Melt (HRM) and a CAPS marker. Distorted segregation in populations segregating for resistance was observed for two SNPs putatively associated with two other genes necessary for expression of resistance. A functional prediction of proteins involved in the resistance mechanisms was performed on genome scaffolds containing the three SNPs of interest. Indeed, 16 full-length Pathogen Recognition Genes (PRGs) were identified around the three SNP markers. In particular, we discovered that two nucleotide-binding site leucine-rich repeat (NBS-LRR) protein-encoding genes were associated closely with the SNP1 marker. The investigation of ZYMV resistance in squash populations and the genomic analysis performed in this work could be useful for better directing the introgression of disease resistance into elite *C. pepo* germplasm.

Keywords: *Cucurbita pepo*; Pathogen recognition genes; SNP markers; squash; ZYMV resistance

INTRODUCTION

Summer squash, *Cucurbita pepo* L. (*Cucurbitaceae*), is among the most widely grown and appreciated vegetable crops in the Mediterranean Basin (Paris 2008). Although summer squash is a short-season crop that is easy to grow, it is highly subject to infection by virus diseases. The most devastating virus disease to summer squash crops is *Zucchini yellow mosaic virus* (ZYMV). First described in Italy by Lisa et al. (1981) and in France by Lecoq et al. (1981), ZYMV is a highly virulent, single-stranded RNA potyvirus that is transmitted by several aphid species in a non-persistent manner (Lisa and Lecoq 1984; Desbiez and Lecoq 1997; Gal-On 2007).

Symptoms of infection by ZYMV in summer squash include stunting of the plant, yellow mosaic, malformation and blistering of leaves, deformed, knobbed fruits and reduced numbers of seeds. Application of chemicals to control the aphid vectors of the virus are largely ineffective in preventing infection (Nameth et al. 1986), and therefore intensive efforts have been made in breeding for resistance to this virus in summer squash (Whitaker and Robinson 1986; Paris 2008). Resistance to ZYMV has been found in several accessions of *Cucurbita moschata* Duchesne, which is sparingly cross-fertile with *C. pepo* (Whitaker and Davis 1962; Whitaker and Robinson 1986). These include an accession from Nigeria named ‘Nigerian Local’ (Munger and Provvidenti 1987), two accessions from Portugal, ‘Menina’ and ‘Bolina’ (Paris et al. 1988) and one from Puerto Rico, ‘Soler’ (Wessel-Beaver 2005).

Brown et al. (2003) reported that a single dominant gene (*Zym-0*) confers resistance to ZYMV in the cross of ‘Nigerian Local’ with the susceptible ‘Waltham Butternut’. In addition, in the cross of the resistant ‘Menina’ with the susceptible ‘Waltham Butternut’, segregation of one dominant gene for resistance, *Zym-1*, was observed (Paris et al. 1988).

However, when the resistance from ‘Menina’ was introgressed into the *Cucurbita pepo* zucchini ‘True French’, segregation into resistant versus susceptible did not conform to a single-gene ratio. The F_2 progeny segregated in accordance with a 45:19 ratio, and the backcross to ‘True French’ segregated in accordance with a 3:5 ratio, suggesting that one of two other dominant genes, *Zym-2* and *Zym-3*, needs to be present together with *Zym-1* for resistance to be expressed (Paris and Cohen 2000). In analyzing additional populations segregating for resistance to ZYMV, Pachner et al. (2011) observed that several other genes could confer ZYMV resistance. Indeed, the ZYMV-susceptible ‘Waltham Butternut’ carries a recessive gene, designated *zym-5*, which is necessary for expression of the resistance conferred by gene *Zym-4*. Moreover, these same authors found that the Puerto Rican pumpkin ‘Soler’ carries a different gene for resistance, designated *zym-6*.

Summer squash hybrids resistant to ZYMV, derived from ‘Nigerian Local’ and ‘Menina’, have been commercially available for 20 years. The breeding process is time-consuming and rife with difficulties inherent to interspecific crossing (Whitaker and Davis 1962; Whitaker and Robinson 1986). Molecular markers can help in dissecting complex resistance traits and in assisting the introgression of resistance into elite germplasm (Pachner et al. 2015). The association between markers and phenotype is dependent on the linkage between the markers and the genes conferring resistance, and the expressivity of these genes. In *Cucurbita*, two SSR markers and one SCAR marker for genes *Zym-0*, *Zym-1*, and *Zym-2* have been described by Pachner et al. (2015). However, various combinations of genes and/or distorted segregation can complicate deciphering the genotypes of resistant plants and possible interactions among genes conferring resistance.

Over the past decade, genetic and genomic resources in *Cucurbita pepo* that can facilitate genetic studies and breeding work in this species have been released (Zraidi et al. 2007; Gong et al. 2008 a,b; Blanca et al. 2011; Esteras et al. 2012). In particular, a dense SNP-genetic

map of *C. pepo* using the Illumina GoldenGate platform was built (Esteras et al. 2012). As single-nucleotide polymorphisms (SNPs) are the most abundant variations in genomes, their identification represents an invaluable resource for both genetic analysis and marker-assisted breeding. Moreover, the draft sequence of the zucchini genome (https://cucurbigene.upv.es/), released recently, allows us to perform investigation of given chromosome regions.

The first goal of the present work was to analyze the inheritance pattern of ZYMV resistance in five segregating populations. In particular, we screened phenotypically and with markers the F_2 populations obtained by crossing the susceptible zucchini 'True French' with its near-isogenic resistant counterpart, Accession 381e, and by crossing Accession 381e with a susceptible summer squash, the *C. pepo* cocozelle 'San Pasquale'. Then we used three backcross populations obtained by crossing three resistant plants of the latter F_2 population with 'San Pasquale', to more closely examine the pattern of inheritance of ZYMV resistance. Our second goal was to identify SNP markers associated with ZYMV resistance, focusing our efforts on the chromosome regions containing these SNPs, in order to identify candidate Pathogen Recognition Genes (PRG) conferring ZYMV resistance.

RESULTS

Genotyping of nine *Cucurbita pepo* accessions

Using the GoldenGate assay on the nine accessions of *Cucurbita pepo*, 134 polymorphic SNPs were identified. Only six SNPs were detected as variations between the two near-isogenic lines, 'True French' (susceptible to ZYMV) and Accession 381e (resistant to ZYMV). Based on the obtained SNP profiles, correlations between accessions were evaluated using a PCA profile (Figure 1). As expected, the three near-isogenic lines, 'True French', Accession 381e, and Accession 968Rb, were clustered tightly and, of the remaining accessions, the zucchini 'Nano Verde di Milano' was closest to them. The four cocozelle accessions, including 'San Pasquale', and the pumpkin 'Tondo Chiaro di Nizza' were distant to the zucchini accessions.

Phenotypic evaluation of segregating populations for ZYMV resistance

The F_2 population derived from the cross 'True French' × Accession 381e segregated for resistance and susceptibility to ZYMV. Of 175 plants, 122 (69.7%) were classified as resistant and, of these, 20 appeared to be highly resistant, similar to Accession 381e. The remaining 53 plants (29.3%) were susceptible, similar to 'True French'. The segregation to resistant and susceptible in this F_2 population fit the one-gene 3:1 ratio and the three-gene 45:19 (Table 1).

Likewise, the F_2 population derived from the cross 'San Pasquale' × Accession 381e segregated for resistance and susceptibility to ZYMV. Of the 96 plants in this population, 62 (64.6%) were resistant and 34 (35.4%) were susceptible. The segregation to resistant and susceptible was in accordance with the 45:19 three-gene and 9:7 two-gene expected ratios (Table 1).

Three resistant plants belonging to the latter F_2 population, numbered 28, 47 and 64, were backcrossed to the susceptible 'San Pasquale' to produce three first-generation backcross populations segregating for resistance to ZYMV. Plant 28 was observed to be highly resistant, similar to Accession 381e, and Plant 47 and Plant 64 were also resistant, but less so than Plant 28. The backcross populations were tested for goodness-of-fit to 1:1, 1:3 and 3:5, one, two and three-gene ratios, respectively, of resistant to susceptible individuals (Table 1).

The backcross population of Plant 28 × 'San Pasquale' (n = 68) had 32 resistant plants and 36 susceptible plants, a result which does not deviate significantly from the three-gene ratio and from the one-gene ratio (Table 1). The backcross population of Plant 47 × 'San Pasquale' (n = 52) had 29 resistant plants and 23 susceptible plants in accordance with the 1:1 ratio. The third backcross population, of Plant 64 × 'San Pasquale' (n = 68) segregated to 19 resistant and 49 susceptible, a result which is in accordance with the 3:5 ratio as well as the 1:3 two-gene ratio.

Molecular characterization of segregating populations for resistance to ZYMV

In order to find markers associated with the genes conferring resistance to ZYMV, we performed an HRM assay for the six identified SNPs in the two near-isogenic lines by the Illumina GoldenGate assay. Out of the six markers used to develop a Real-Time PCR-HRM assay, three (SNP1, SNP2 and SNP3) produced a clear melting curve and were used for further analyses. HRM assays for SNP1, SNP2 and SNP3, carried out on the five segregating populations for resistance to ZYMV, allowed us to discriminate between plants that were homozygous or heterozygous for these three SNPs by melting curve analysis, as shown in Supplementary Figure 1. For brevity, the SNP1, SNP2, and SNP3 loci will hereafter be symbolized with *A*, *B*, and *C* designations, respectively.

HRM analysis performed on the 'True French' × Accession 381e F_2 progeny revealed

that, out of 174 analyzed plants, 130 had allelic condition *A/-* and 44 plants had *a/a*. This segregation between the *A/-* and *a/a* individuals fits the 3:1 ratio (chi-square = 0.008, *P* = 0.93). As regards the 'San Pasquale' × Accession 381e F_2 population, out of 91 analyzed plants, 68 were *A/-* and 23 *a/a*. Also in this case, segregation to *A/-* and *a/a* fits the 3:1 ratio (chi-square = 0.004, *P* = 0.95) as showed in the Table 2.

By converting SNP1 into a CAPS marker on a sample of 167 'True French' × Accession 381e F_2 plants, we were able to confirm the results obtained using the HRM assay. The PCR amplicon of 400 bp was digested with the BglII restriction enzyme producing two fragments of 200 bp only in the susceptible samples, whereas the heterozygous samples produced both undigested and digested fragments (Supplementary Figure 2). The results obtained from the CAPS marker analysis also fit with a 3:1 ratio (Table 2).

Similarly, for the SNP2 and SNP3 loci, there were no significant deviations from expected 3:1 ratio in the F_2 populations (Table 2). This was the case for both, the F_2 'True French' × Accession 381e and the F_2 of 'San Pasquale' × Accession 381e.

The three BC_1-F_2 populations obtained backcrossing the F_2 28, 47 and 64 plants of 'San Pasquale' × Accession 381e on 'San Pasquale' were also assessed by HRM assay. Plant 28 was heterozygous at all three loci, *A/a B/b C/c*. Plant 47 was heterozygous at the first locus but showed homozygous recessive alleles at other two, *A/a b/b c/c*. Plant 64 had genetic constitution *A/a B/B C/C*. No deviation from the 1:1 expected ratio for *A/-* versus *a/a* occurred in the backcross populations, but this was not the case for *B/-* versus *b/b* and *C/-* versus *c/c* (Table 2).

Distorted segregation in two analyzed populations

To determine whether or not distorted segregation occurred in the segregating populations, we tested goodness-of-fit for each of them (Table 4 and Supplementary Tables 1

and 2). The F_2 population of 'True French' × Accession 381e (n = 171) deviated highly significantly (chi-square = 88.924, df = 26, P = < 0.001) from expectation of random segregation, and indicating a degree of co-segregation of SNP2 and SNP3 (*B* and *C*). Also the backcross progeny derived from Plant 28 (n = 64) deviated very highly significantly from the expected distribution among the eight possible genotypes expected from random segregation at loci *A*, *B*, and *C* ($\chi 2$ = 37.000, df = 7, P = 4.690 e-6), with a pronounced excess of classes *A/a b/b c/c* and *a/a B/b C/c*, that highlighted a SNP2 and SNP3 co-segregation trend (Figure 2; Supplementary Table 2). In Table 4 we summarized the chi-square, *P value* of each five segregating populations related to the distorted segregation of analyzed populations.

Co-segregation data analysis

In order to assess the tendency of the markers to co-segregate with the observed resistance or susceptibility to ZYMV, the percent of co-segregation between each marker and reaction to ZYMV was calculated (Table 3). For the F_2 population derived from the cross 'True French' × Accession 381e, 122 plants were classified as resistant and 53 as susceptible (Table 1); the SNP1 marker was exhibited by 130 plants (Table 2), 117 (90%) of which were resistant. In addition, the SNP marker was absent from 5 of the 122 resistant plants, which is 4% of the total resistant plants. For the F_2 population derived from the cross 'San Pasquale' × Accession 381e, 62 plants were classified as resistant and 34 as susceptible (Table 1); the SNP1 marker was exhibited by 68 plants, 54 (79%) of which were resistant.

In the BC_1 populations, the co-segregation between marker SNP1 and resistance averaged 79%, indicating a fairly strong linkage SNP1 and the ZYMV resistance trait. Specifically, the minimum percent of co-segregation into BC_1 populations was represented by the backcross population derived from Plant 64 × 'San Pasquale' (allelic configuration *A/a B/B C/C*), consisting of 68 plants, 19 of which were classified as resistant and 49 as susceptible. The

SNP1 marker was exhibited by 28 plants, 15 (53%) of which were resistant. Meanwhile the observed co-segregation in the Plant 28 × 'San Pasquale' population, which consisted of 36 plants that exhibited SNP1, was 92% (33 resistant plants).

Co-segregation for SNP2 and SNP3 was variable, but over 60% in the F_2 populations. Such findings suggest that the association of those markers to resistance is not high, either because they are not very close to the loci conferring ZYMV resistance and/or because the loci modify only slightly the ZYMV resistance. Assuming that three separately inherited genes contribute to ZYMV resistance and that A, B, and C are putatively dominant to a, b, and c (where A/a, B/b and C/c corresponds to SNP1, SNP2 and SNP3, respectively), each gene does not contribute equally to the phenotype, highlighting that epistatic or additive interactions can occur among such genes modifying the segregation *ratio*. Moreover, if some gametes are transmitted to the next generation at a lower frequency, the distorted genetic segregation could potentially complicate the interpretation and the further introgression of the genes for ZYMV resistance into *Cucurbita pepo* germplasm.

In order to calculate the overall relationship between phenotypic and genotypic results, we used the Mantel test on data obtained from 'True French' × Accession 381e and 'San Pasquale' × Accession 381e F_2 populations. In both populations the $P < 0.0001$. P was calculated using the distribution of r (AB) = 0.186 in the 'True French' × Accession 381e and 'San Pasquale' × Accession 381e F_2 populations, estimated on 10,000 permutations. The results obtained allowed us to confirm the close correlation existing between dissimilarity matrices, based on the Euclidean distance and originated by phenotypic and genotypic datasets that separated individuals belonging to the two F_2 segregating populations in three groups (Figure 3). Moreover, this allows us to affirm that the number of plants showing an unexpected correlation between genotypic and phenotypic data is statistically not significant.

Candidate Pathogen Recognition Genes for ZYMV resistance

In order to identify genes physically close to our markers, a functional prediction of proteins involved in the resistance mechanisms was performed. The physical positions of markers associated with ZYMV resistance on the *Cucurbita pepo* draft genome were identified (Supplementary Table 3).

Unigene genomic loci containing the SNP1, SNP2 and SNP3 markers are located on Scaffolds 8, 21 and 22, respectively. The coding sequences (CDS) predicted, on a total of ~10 Mb genome sequence, were translated and analyzed by InterProScan to identify the protein domain composition. All the predicted protein-encoding genes (1398) were functionally annotated and categorized in families. The predicted loci of three unigenes associated with SNP1, SNP2 and SNP3 markers codify for a DEAD/DEAH_box_helicase_dom (ID: IPR011545), Coiled-coil-dom_prot_109_C (ID: IPR006769), and Pectate_lyase_SF_prot (ID: IPR024535), respectively. A total of 17 pathogen recognition genes (PRGs) were identified on the three scaffolds analyzed, of which 16 were full-length. On Scaffold 8, the highest number (12) of PRGs were annotated, in particular 8 RLKs (Receptor-Like Kinases), 2 CNLs (Coiled coil, Nucleotide binding sites Leucine-rich_repeats), 1 RLP (Receptor like-Protein) and 1 TN (Nucleotide binding site) were characterized. Analyzing in major detail the genomic region surrounding SNP1, 2 CNLs closely associated with this marker were evident (8 and 22 Kbp) as shown in Figure 3. The physical closeness between these CNLs and SNP1 suggest that such genes could be associated with ZYMV resistance function (Figure 4). On Scaffold 21, only one RLK was predicted and on Scaffold 22 three RLKs and one RLP were predicted, indicating that there is a low number of PRGs.

DISCUSSION

The process of introduction of pathogen resistances in crop species, particularly those resistances that are under complex control, is time-consuming and the resistances are not always stable over time. Therefore, the most reasonable approach towards the introduction of different resistance genes into one genotype is a step-wise combination of phenotypic and genome-assisted selection. Molecular genetics and modern biotechnology techniques can be very useful for capturing the entire variation of genetic determinants (Ercolano et al. 2012; Pachner et al. 2015).

The main goal of this study was to find molecular markers associated with genes conferring ZYMV resistance, in order to make easier, faster and economically sustainable the introgression of these genes into summer squash. We evaluated the inheritance of ZYMV resistance in five *Cucurbita pepo* segregating populations, using the same source of resistance described by Paris et al. (1988) and Gilbert-Albertini et al. (1993). The phenotypic assays, performed on the two F_2 populations 'True French' × Accession 381e and 'San Pasquale' × Accession 381e, and three derived by backcrossing individual resistant plants from the F_2 population of 'San Pasquale' × Accession 381e, to the susceptible parent, 'San Pasquale', allowed us to identify resistant and susceptible plants. The observed segregation in the two F_2 and in the three BC_1 segregating populations were in accordance with both single and three complementary dominant genes expected *ratios* (Paris and Cohen 2000; Pachner et al. 2015).

Segregation for resistance to ZYMV, studied at both the phenotypic and genotypic levels, indicated clearly that a major genes, *Zym-1*, is essential for expression of resistance. The main role of *Zym-1* in ZYMV resistance was confirmed by a high co-segregation percentage between SNP1 and resistant phenotypes in analyzed populations, and supported by Mantel

289 test analysis conducted on the two F_2 populations. Of the other two genes, the presence of at
290 least one is reported necessary for resistance to be expressed (Paris et al. 2000). Their
291 contribution varying from the 61% to the 76% in F_2 populations (Table 3). Both F_2s
292 segregated in accordance with the 45:19 three complementary-gene ratio. However, SNP2
293 and SNP3 co-segregation deviated significantly from expected ratio of resistant-to-
294 susceptible. Clearly, the particular genomic regions in which they are located are more
295 subject to segregation distortion and such occurrence can misleading interpretation and
296 breeding selection.

297 Pachner et al. (2015) indicated that the combined effect of the seven genes identified as
298 conferring ZYMV resistance would require for maximal expression of resistance. Various
299 combinations of these genes would result in a phenotypic spectrum of resistance expression,
300 though the presence of *Zym-1* is essential. Gomez et al. (2009) reported that more than 50%
301 of resistance traits to plant viruses are dominant, 35% are recessive, and the remainder have
302 more complex pattern involving incomplete dominance or dosage dependency.

303 The resistance analyzed in this work requires additional genes for its enhancement and for
304 ensuring its continued expression through late stages of plant development (Pachner et al.
305 2015). In pepper, important epistatic effects between a major and three minor QTLs (with or
306 without additive effects) in resistance to TMV and *Phytophthora capsici* were detected
307 (Lefebvre and Palloix 1996). Extensive variation in basal expression of the same R-gene
308 present in accessions collected from different environments was found in Arabidopsis
309 (MacQueen and Bergelson 2016). Virus spread can be limited by temperature (Valkonen et
310 al, 2008) and by aged vascular tissue (Collum et al. 2016). Inducible defense is a cost-saving
311 strategy, and may occur only in conditions where it confers a fitness benefit to the individual
312 (Cipollini 2008).

313 The detected segregation anomalies in our genetic populations can account, at least in

part, for the unexpectedly high level of genetic similarity observed among ZYMV-resistant cultivars of *Cucurbita pepo* (Formisano et al. 2010). Segregation distortion can be highly variable in its extent and in the number of loci affected. Most segregation distortion regions are specific to one population, but preferential regions can be shared among populations. In barley reciprocal crosses, several shared distorted regions were highlighted (Bélanger et al. 2016). A quasi-linkage phenomenon has been reported, too, in the F_2 of a cross between a dessert watermelon and a citron watermelon (Levi et al. 2003). In this investigation, non-homologous linkage groups behaved as one comprehensive linkage group, and suggested that this phenomenon might be the result of strong affinity among non-homologous chromosomes or chromosome regions, causing them to pass to the same pole during cell division.

Molecular genetic studies can be useful for monitoring the introgression of desirable traits and for overcoming related difficulties. Besides the selection pressure for or against some allelic combinations, our molecular results showed a stronger co-segregation of SNP1 with ZYMV resistant phenotype. Phenotype-genotype co-segregation was maintained up to 80% for SNP1, but was lower for SNP2 and SNP3. The assembling of multiple desirable genes into a single accession could be accelerated using genotyped advanced breeding populations (Ye and Smith 2008). Molecular-based selection power is likely to increase using marker developed on the sequence of the genes of interest (Xu and Crouch 2008). By exploring genomic regions containing SNP1 markers, we identified proteins belonging to the CNL class, suggesting association of our SNP1 with a resistance gene. Furthermore, we noticed that SNP1 was localized in a gene coding RNA helicases. Xu et al. (2013) reported the involvement of RNA helicases in response to stress, and in plant growth and development. The loss of function in a helicase gene has a deleterious effect on viral ToMV infectivity (Ishibashi et al. 2014). RLP and RLK proteins, potentially involved in pathogen recognition, were also found on the scaffolds to which belong the SNP2 and SNP3. Such proteins relay on

339 extracellular signals to initiate an intracellular basal defense response, and some of them
340 interact with virus to suppress host defense, or to potentiate virus infection (Sakamoto et al.
341 2012)

342 The main role of *Zym-1* in response to ZYMV infection in squash has been highlighted. A
343 close relationship of SNP1 with a CNL and an ATP-dependent RNA helicase is consistent
344 with the association of SNP1 with one gene conferring resistance, *Zym-1*. The HRM marker
345 developed for SNP1 and the validating CAPS marker are available for marker assisted
346 selection. Moreover, awareness of distorted segregation as well as the future elucidation of
347 the underlying mechanism could aid breeders in designing future appropriate crossing
348 schemes.

349

MATERIALS AND METHODS

Plant materials

Nine summer squash accessions derived from three cultivar-groups, Zucchini, Cocozelle, and Pumpkin (Paris 1986), were used for the Illumina GoldenGate genotyping assay. These included four zucchini accessions, 'Nano Verde di Milano', 'True French', and two near-isogenic lines of 'True French', one being Accession 381e, which is resistant to ZYMV (Paris and Cohen 2000), and the other being Accession 968Rb, which is resistant to powdery mildew (Cohen et al. 2003). The other five accessions were four cocozelles, 'San Pasquale', 'Romanesco', 'Ortolana Di Faenza', and 'Bianca di Trieste', and one pumpkin, 'Tondo Chiaro di Nizza'. Seeds of 'True French' and its two near-isogenic lines were from the germplasm collection maintained at the Newe Ya'ar Research Center (Ramat Yishay, Israel) (Paris 2001) and seeds of the other six cultivars were kindly provided by La Semiorto Sementi S.r.l. (Sarno, Italy).

The ZYMV-susceptible 'True French' (TRF) was crossed with its near-isogenic ZYMV-resistant counterpart, Accession 381e (Paris and Cohen 2000), to obtain an F_2 population. Accession 381e was also crossed with the ZYMV-susceptible 'San Pasquale' in order to produce another F_2, and three resistant plants selected from this F_2 were then backcrossed to 'San Pasquale', to obtain three first-generation backcross populations.

In vivo assay of resistance to ZYMV in segregating populations

Seeds of parental and filial generations were sown in multi-cellular trays consisting of 4 cm diameter pots filled with peat, one seed per pot. Subsequently, the seedlings were transplanted to pots 15 cm diameter and were grown in an insect-free glasshouse at 22-24°C using supplemental lighting to maintain 12-hour photoperiod, at the Council for Agricultural

Research and Agricultural Economy Analysis (CREA) in Rome. Each individual plant was numbered.

An isolate of ZYMV from a naturally infected plant of field-grown summer squash was used for experimentation. This isolate caused the typical symptoms of zucchini yellow mosaic, including yellow mosaic, vein banding, blistering and malformation of leaves. Symptomatic leaves of artificially infected zucchini plants were crushed, and the raw juice was extracted at a ratio of 1:10 w/v in 0.1 M phosphate buffer pH 7.2. Cotyledons of plants to be tested were sprinkled with the abrasive powder "Celite" and inoculated with approximately 20 µl of the diluted extract, and subsequently washed with distilled water. Symptoms were observed and recorded from 6 to 22 days post inoculation (dpi), when the plants had developed 4–5 true leaves. Parental as well as F_1 plants were used as resistant and susceptible controls. According to the symptoms observed at 6, 12 and 22 days past inoculation (dpi) and based on descriptions and illustrations by Paris and Cohen (2000), the plants were classified as resistant or susceptible. Some resistant plants, at 22 dpi, were nearly asymptomatic, having few tiny yellow dots on their leaf laminae, similar to the resistant parent, Accessions 381e. Most of the resistant plants, though, showed yellow spots and vein banding of the leaf laminae. Susceptible plants exhibited leaf deformation and yellow mosaic of the leaf laminae. To ensure that the nearly asymptomatic plants were truly resistant and not escapes from virus inoculation, a serological ELISA test was performed on all plants (data not shown).

Molecular analysis

Total genomic DNA from all plant samples was extracted using the protocol described by Fulton et al. (1995). The DNA amount was quantified spectrophotometrically by NanoDrop ND-1000 Spectrophotometer (Nano Drop Technologies) and the quality was checked

electrophoretically with a 1.2% agarose gel staining using a SYBR® Safe DNA Gel Stain (Takara).

The Illumina GoldenGate genotyping assay was performed on the nine accessions of *C. pepo*. The GoldenGate genotyping assay was conducted on one set of 384 SNPs, as described in Esteras et al. (2012) at Centro Nacional de Genotipado (CEGEN-ISCIII, CRG-Node, Barcelona, Spain).

The High Resolution Melt (HRM) Real-Time PCR mixture was prepared using 15–25 ng of DNA, 0.6 U of exTaq TM (Takara), 20 mM of Mg^{2+}, 2.5 µl of 10X exTaq Buffer 10 mM, 2.5 mM dNTPs and primers were mixed to a final concentration of 10 uM. The intercalating dye was EvaGreen (Biothium) at final concentration of 0.5X. Real-Time PCR HRM reactions were performed on Rotor-Gene 6000™ (Corbett Research, Cybeles, Thailand). The Real-Time PCR profile was comprised of one initial cycle of 30 s at 95°C and followed by 40 cycles of 10 s at 95°C, 30 s at 58–60°C, and 30 s at 72°C. After Real-Time PCR amplification, HRM was performed using a melting profile from 70°C to 90°C rising at 0.1°C per sec. The specificity of primers used in the experiment was confirmed by a single amplicon melting analysis. DNA of resistant and susceptible parents as well as their F_1s was used as controls. Data from HRM analysis was determined using Rotor-Gene 6000 series 1.7 software. All of the analyzed data were obtained according to the manufacturer's protocol (HRM guide https://www.qiagen.com/it/shop/automated-solutions/pcr-instruments/rotor-gene-q/).

The CAPS analysis consisted of a PCR reaction and subsequent digestion with BglII restriction enzyme that recognizes A^GATCT sites. PCR amplification was carried out in a final volume of 25 µl, Buffer 1X, 0.1 mM dNTPs, 0.2 µM, Taq polymerase 0.5 U, and 0.2 µM each primer, DNA 25-35 ng. Amplification was performed under the following program: 30 s at 95°C; 40 cycles of 10 s at 95°C, 30 s at 57°C and 30 s at 72°C; extra extension for 1

min and 30 s at 72°C. Amplified products (15 µl) were treated for 3 hours at 37°C with 1 U of BglII restriction enzyme in a final volume of 25 µl. Polymorphism was detected by separating the whole volume of treated DNA on 2% agarose gel (1X Tris-Acetate EDTA) containing SYBR® Safe DNA Gel Stain (Takara), and visualizing it under a UV transilluminator. A $1Kb^{+}$ ladder fragment size markers was used as a control (Invitrogen).

Statistical analysis

Principal Component Analysis (PCA) was performed from genotyping data obtained by the Illumina GoldenGate assay using GenAlEx 6.41 (http://biology-assets.anu.edu.au/GenAlEx/Welcome.html) (Peakall and Smouse 2012) and MEGA7 software (www.megasoftware.net) (Tamura et al. 2011).

Segregation to resistance and susceptibility to ZYMV in the F_2 and backcross populations was subjected to chi-square analysis. In addition, the results from the HRM analysis for all molecular markers were subjected to chi-square analysis.

Furthermore, a Mantel test (Mantel 1967) was executed between phenotypic and genotypic data to find a correlation between the two trends. In order to obtain the phenotypic and genotypic matrices, the Ascendant Hierarchical Coefficient (HAC) was performed by the XLStat tool (http://www.xlstat.com/en) (Addinsoft 2007). MEGA7 tool was used to obtain the dendrograms based on the Euclidean distance between the matrix distributions. The Pearson's correlation coefficient was calculated for the two matrices through a correlation/association test (Mantel test).

Identification of candidate Pathogen Recognition Genes

An in-house pipeline to identify Pathogen Recognition Genes (PRGs) physically close to our SNP markers was used. The queried squash scaffolds were identified by mapping

GoldenGate probe sequences to the draft of the *Cucurbita pepo* genome sequence (version 3.2) (http://cucurbigene.upv.es) using BLASTn (E-value 1e-10). To identify the PRG coding sequences located on scaffolds associated with our markers, GENSCAN software was used (Burge et al. 1988). The identified PRG coding sequences were translated into peptides in order to verify the presence of conserved domains characteristic of resistance proteins (CNL: Coiled coil, Nucleotide-binding site, Leucine-rich repeat; TNL: Toll/interleukin-1 receptor, Nucleotide-binding site, Leucine-rich repeat; RLP: Receptor Like-Protein; RLK: Receptor Like-Kinase). The identified proteins were further analyzed using InterProScan software version 5 (Zdobnov and Apweiler 2001).

Acknowledgements

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570 ## Figures and Tables legend

571

Table 1 Segregation to resistant (R) and susceptible (S) to Zucchini yellow mosaic virus in two F_2 and three BC_1 populations of summer squash plants, *Cucurbita pepo*

Population	Number of plants			Expected ratio	Chi-square	*P*
	Total	R	S			
F_2, TRF (S) × 381e (R)	175	122	53	45:19	0.030	0.86
				9:7	12.89	<<0.01
				3:1	2.608	0.11
F_2, SPQ (S) × 381e (R)	96	62	34	45:19	1.510	0.22
				9:7	2.709	0.09
				3:1	5.556	0.02
Total F_2	271	184	87	45:19	0.533	0.46
				9:7	14.94	<<0.01
				3:1	7.293	<0.01
BC_1-F_2 (F_2-28 × SPQ)	68	32	36	3:5	2.651	0.10
				1:3	17.65	<<0.01
				1:1	0.25	0.62
BC_1-F_2 (F_2-47 × SPQ)	52	29	23	3:5	7.405	<0.01
				1:3	26.26	<<0.01
				1:1	0.69	0.41
BC_1-F_2 (F_2-64 × SPQ)	68	19	49	3:5	2.651	0.10
				1:3	0.314	0.57
				1:1	13.24	<<0.01
Total BC_1	188	80	108	3:5	2.048	0.16
				1:3	30.89	<<0.01
				1:1	4.170	0.04

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574 **Table 2** Segregation for three SNP markers in two F_2 and three BC_1 populations of summer squash plants,

575 *Cucurbita pepo*

Population	Marker (linkage)	Number of plants			Expected ratio	Chi-square	*P*
		Total	Dominant allele	Recessive homozygous			
F_2, TRF (S) × 381e (R)	SNP1 CAPS	167	123	44	3:1	0.162	0.70
	SNP1	174	130	44	3:1	0.008	0.93
	SNP2	174	138	36	3:1	1.724	0.19
	SNP3	173	138	35	3:1	2.098	0.15
	3 SNPs	174	130	44	45:19	1.614	0.20
F_2, SPQ (S) × 381e (R)	SNP1	91	68	23	3:1	0.004	0.95
	SNP2	91	66	25	3:1	0.297	0.58
	SNP3	88	66	22	3:1	0.000	1.00
	3 SNPs	91	68	23	45:19	0.85	0.36
BC_1-F_2, (F_2-28 × SPQ)	SNP1	65	36	29	1:1	0.754	0.39
	SNP2	64	31	33	1:1	0.063	0.80
	SNP3	65	29	36	1:1	0.754	0.39
	3 SNPs	65	36	29	3:5	8.87	0.003
BC_1-F_2, (F_2-47 × SPQ)	SNP1	49	21	28	1:1	1.000	0.32
	SNP2	51	0	51	--	--	--
	SNP3	51	0	51	--	--	--
	3 SNPs	49	21	28	3:5	0.60	0.44
BC_1-F_2, (F_2-64 × SPQ)	SNP1	60	28	32	1:1	0.267	0.61
	SNP2	68	68	0	--	--	--
	SNP3	68	68	0	--	--	--
	3 SNPs	60	28	32	3:5	2.15	0.14

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578 **Table 3** Co-segregation of resistance and susceptibility to Zucchini yellow mosaic virus with molecular markers

579 putatively linked to the genes conferring resistance

Population	Resistance Gene	Marker	Population size	Co-segregation (%)
F_2, TRF (S) × 381e (R)	*Zym-1*	SNP1 CAPS	167	86
	Zym-1	SNP1	174	90
	Zym-2	SNP2	174	72
	Zym-3	SNP3	173	70
F_2, SPQ (S) × 381e (R)	*Zym-1*	SNP1	91	79
	Zym-2	SNP2	91	76
	Zym-3	SNP3	88	61
BC_1-F_2 (F_2-28 × SPQ)	*Zym-1*	SNP1	65	53
	Zym-2	SNP2	64	28
	Zym-3	SNP3	65	28
BC_1-F_2 (F_2-47 × SPQ)	*Zym-1*	SNP1	49	91
	Zym-2	SNP2	51	0
	Zym-3	SNP3	51	0
BC_1-F_2 (F_2-64 × SPQ)	*Zym-1*	SNP1	60	92
	Zym-2	SNP2	68	52
	Zym-3	SNP3	68	59

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582 **Table 4** Distorted segregation in the F_2 and BC_1 segregating populations. A distorted segregation of SNPs was
583 observed in the BC_1 Plant 28 × 'San Pasquale' and F_2 'True French' × Accession 381e populations. These two
584 populations are labelled in yellow.

Population	Total Samples	Degree of freedom	Chi-square	*P*
F_2, TRF (S) × 381e (R)	171	26	88.924	<< 0.001
F_2, SPQ (S) × 381e (R)	87	26	31.825	0.199
BC_1-F_2, (F_2-28 × SPQ)	64	7	37.000	< 0.001
BC_1-F_2, (F_2-47 × SPQ)	49	1	1.000	0.317
BC_1-F_2, (F_2-64 × SPQ)	60	1	0.267	0.606

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Figure 1 PCA analysis on genotyping data obtained by Illumina GoldenGate assay using a panel of 384 SNPs. Genetic relationships between nine *Cucurbita pepo* cultivars: True French (TFR), 381e, 968Rb, Nano Verde di Milano (NVM), Romanesco (ROM), Ortolana di Faenza (ODF), San Pasquale (SPQ), Bianca di Trieste (BDT), Chiaro di Nizza (TON). The PCA analysis was performed using GenAlEx 6.41 and MEGA5 software. The three isogenic lines True French (susceptible to ZYMV), Accession 968Rb (susceptible to powdery mildew) and Accession 381e (resistant to ZYMV) polled together

Figure 2 Distorted segregation in BC_1 (28 × 'San Pasquale)' population.

Percentage of observed (blue) and expected (orange) genotypic classes in 28 × 'San Pasquale' population is reported . An excessive representation of AaBbCc, Aabbcc, aaBbCc, aabbcc individuals was detected. The calculated chi-square between the expected and observed genotypic classes has a *P value* lower of 0.01, confirming a not random gametes segregation.

Figure 3 A Mantel test to relationship phenotypic and genotypic results.

Sample clustering, based on phenotypic datasets, in three classes of evaluation of F_2 'True French' × Accession 381e (a) and F_2 'San Pasquale' × Accession 381e (d). Sample clustering, on genetic dataset, in 23 out of the 27 expected allelic configurations in segregation of three genes in the crosses F_2 'True French' × Accession 381e (b) and F_2 'San Pasquale' × Accession 381e (e). Mantel test output obtained from the correlation between phenotypic and genotypic matrices of F_2 'True French' × Accession 381e (c) and F_2 'San Pasquale' × Accession 381e (f) represent the samples distribution (blue dots) into the three phenotypic and twenty-seven genotypic classes. The x-axis and y-axis represent the phenotypic and genotypic data matrix, respectively. In both populations, the p-value was < 0.0001

Figure 4 High-resolution GENSCAN analysis of the genomic region surrounding SNP1.

614 Overview of predicted genes (green and blue arrows) and organization of the SNP1-region (~50 Kbp) of the *C.*
615 *pepo* Scaffold000008. The SNP1 position (red spot) on Unigene9577, the exon-intron structure of predicted
616 genes (black boxes and lines, respectively), and the two CNL loci (blue arrows) are shown

Figure 1

Click here to download Figure Fig.1.tif

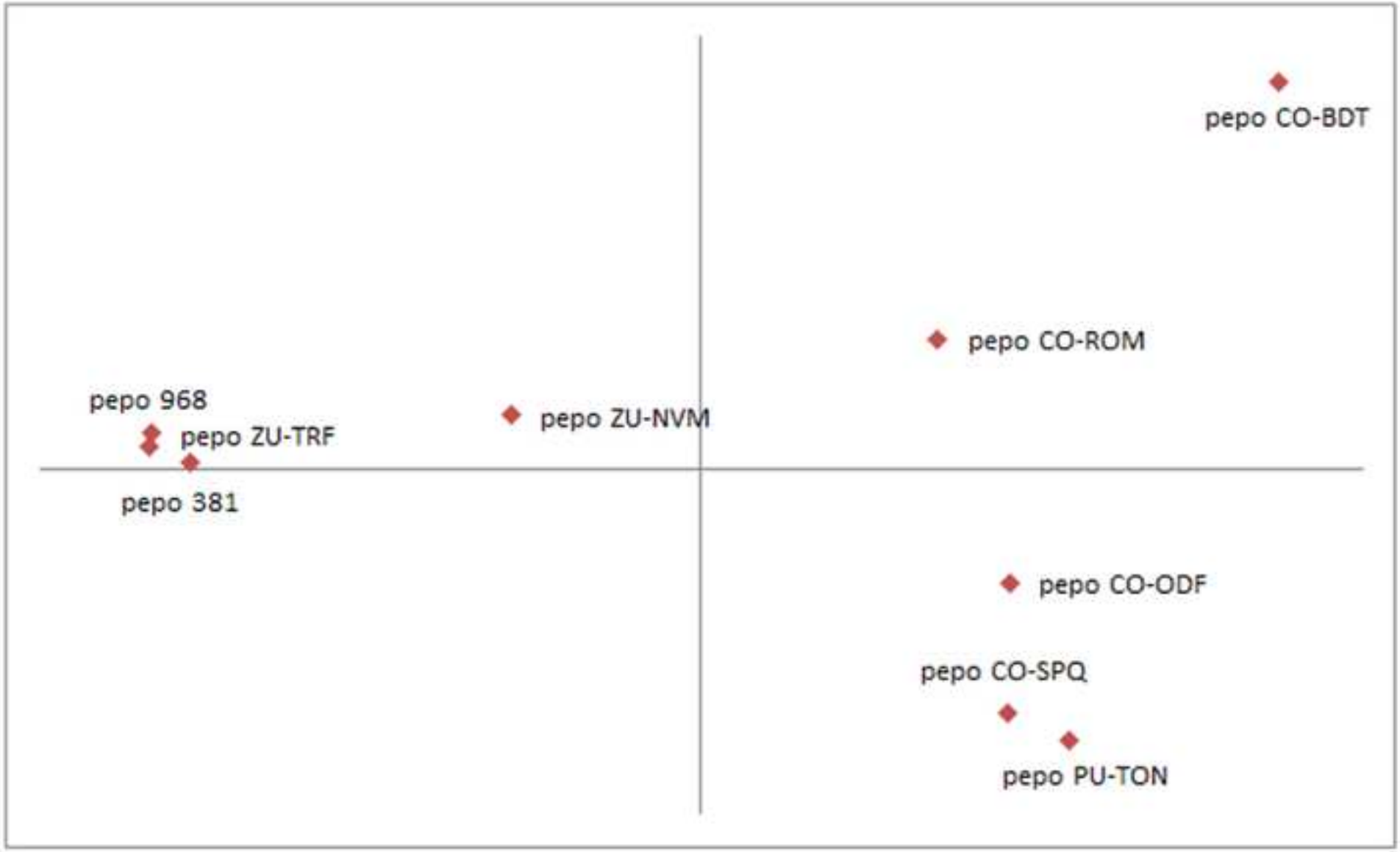

Figure 2

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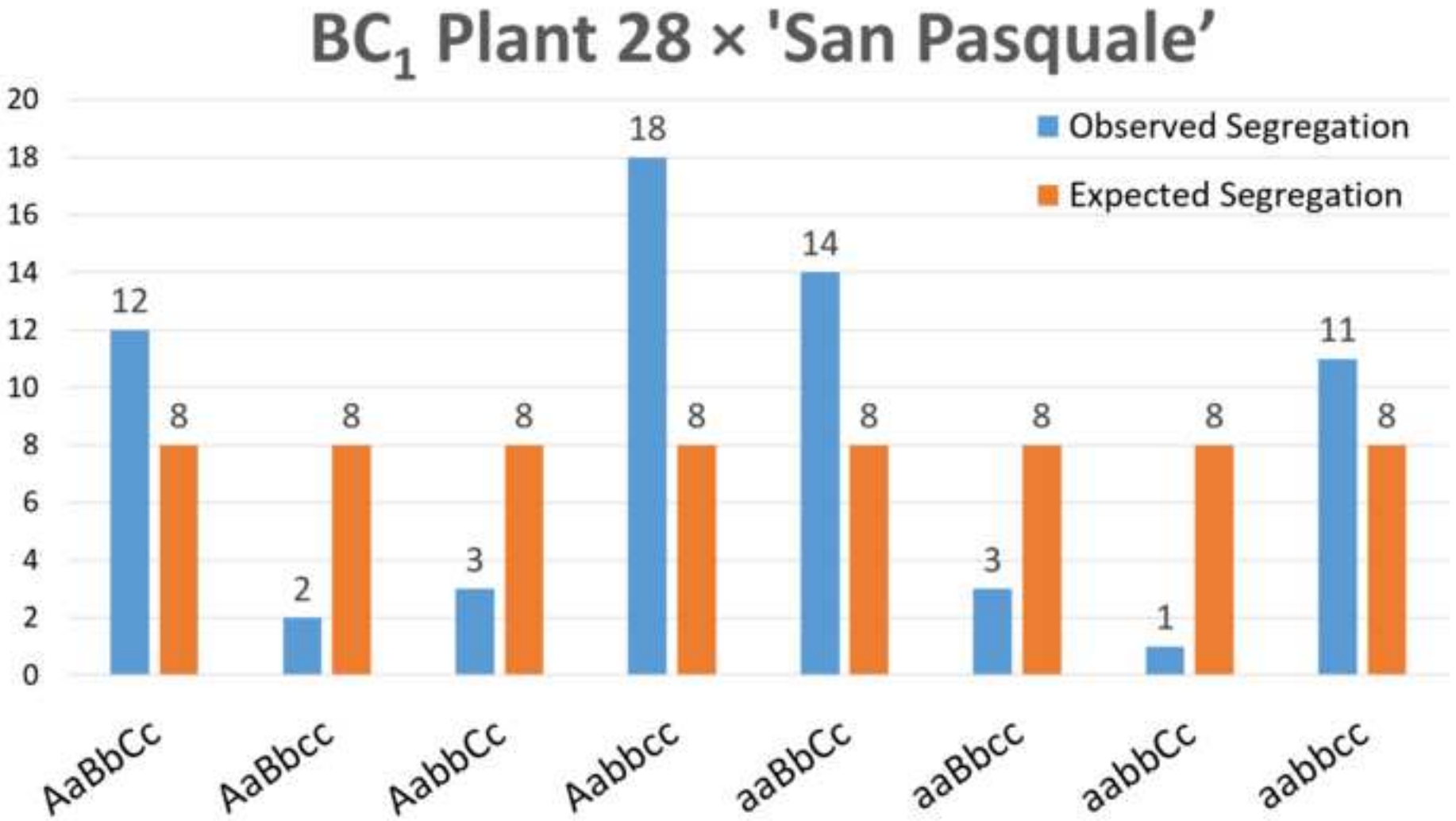

Figure 3

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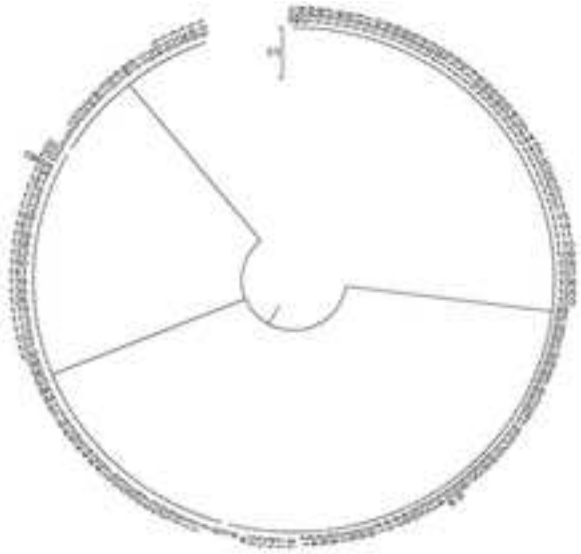
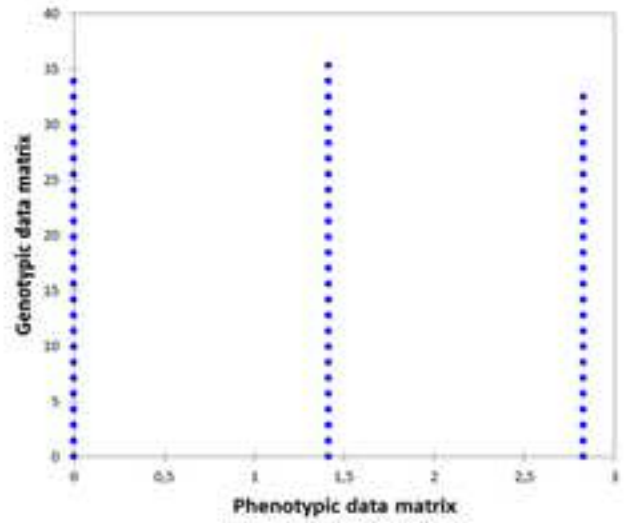


a

b



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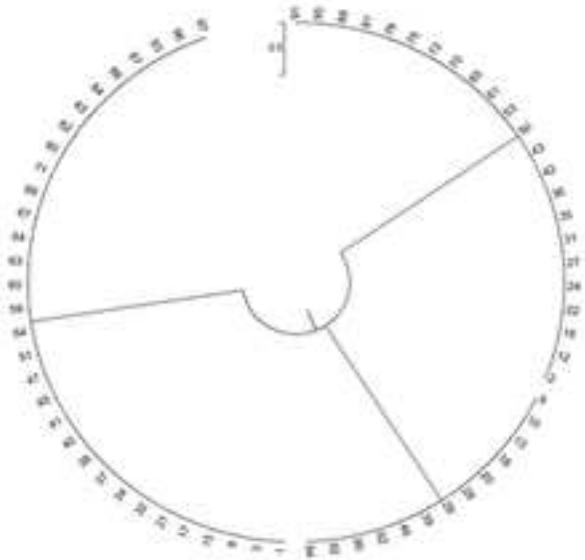

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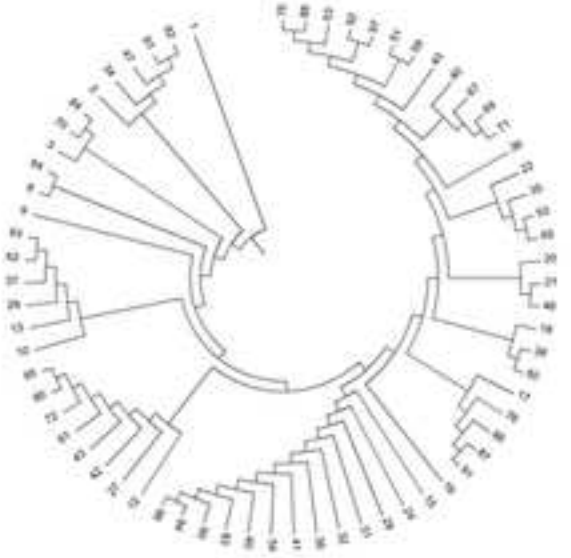

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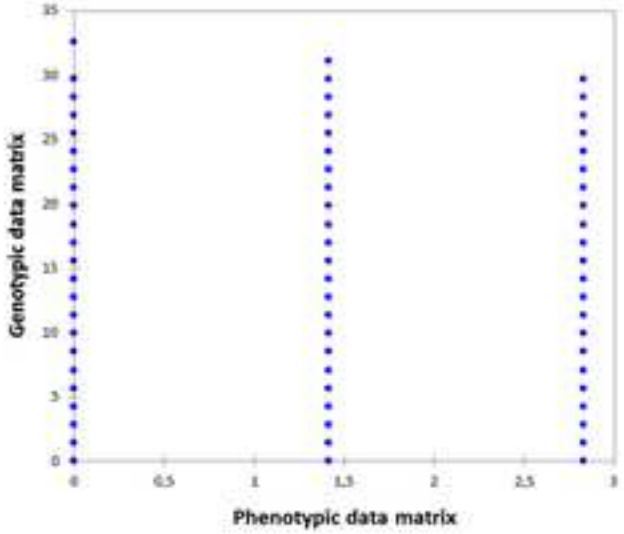

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Figure 4

Click here to download Figure Fig.4.tif

Supplementary tables