FC242 (PI 687276), a Sugar Beet Population with Enhanced Disease Resistance Derived from a Crop Wild Relative, WB242.

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ABSTRACT

Using marker assisted selection, a germplasm with a high frequency of resistance to sugar beet cyst nematode and rhizomania has been selected out of a single plant cross developed to verify the resistance to sugar beet cyst nematode conditioned by the HsBvm-1 gene. Seventeen F_3 families from a mapping cross, homozygous for markers linked to the Rz1 gene (conferring resistance to Beet necrotic yellow vein virus) and the HsBvm-1 gene (conferring resistance to Heterodera schachtii), have been increased and released as an F_5 population. This population also has the potential to contain the Pm gene for resistance to powdery mildew but was not screened for this. Nine F_3 families were chosen at random and greenhouse testing was completed to verify the usefulness of the HsBvm-1 gene marker in a pre-breeding program. Eight of

the 9 families performed as predicted by the marker. One family chosen as segregating for the marker showed the phenotype of a susceptible population. Further experiments showed a distorted segregation ratio in this F_3 family. HsBvm-1, the SNP marker linked to the HsBvm-1 gene is useful in a pre-breeding program and allows an increased efficiency in selecting a population of enhanced frequency of the HsBvm-1 gene before the line is taken for field testing.

Additional Key Words: *Beta vulgaris*, crop wild relatives, host plant resistance, introgression of wild relatives, pre-breeding, *HsBvm-1* (the single gene conferring cyst nematode resistance), HsBvm-1 (the marker linked to the gene conferring cyst nematode resistance), *Rz1, Rz2* (single genes conferring resistance to rhizomania), Rz1, Rz2 (markers lined to the single genes conferring resistance to rhizomania).

INTRODUCTION

Perhaps the most devastating soil-borne pest of sugar beet (*Beta vulgaris* subspecies *vulgaris*, L) worldwide is the sugar beet cyst nematode (SBCN) (*Heterodera schachtii* Schm.) (Dewar and Cooke, 2006; Gray, 2009;Biancardi et al., 2010; McCarter, 2009). It has been reported throughout the United States and worldwide where sugar beet is cultivated (McCarter, 2009; Cooke, 1992; Gray, 2009). In the major areas of sugar beet production, often 10 to 25% of the acreage is infested with this pest (Lange and de Bock, 1994). This pest is hosted by over 80% of the species in the *Amaranthacea* and *Brassicaceae* families (Steele, 1965). The damage is caused by the nematode destroying the tap root, diminishing the ability of the plant to absorb water, and feeding on the plant cytoplasm in the infested root cells (Steele, 1984). In heavily infested fields, crop yields can be depressed by up to 80%. Current management systems rely on Telone II™, Temik™, or other nematicides, crop rotations, trap crops, and, more recently, resistant and tolerant varieties (Franc et al., 2001; Barker and Koenning, 1998; Panella and Lewellen, 2007). The nematicides/fumigants have been removed from the market in some states and are in danger of being removed in others because of environmental concerns, which makes other management tools more important. , which often are in heterozygous for this resistance

Rhizomania (caused by *Beet necrotic yellow vein virus* – BNYVV) is now in every major production area of the United States and present in most sugar beet growing areas worldwide (Pavli et al., 2011). This disease can cause major losses in sugar beet root yield, reduction in the concentration of sucrose in the root, and reduction of juice quality (which interferes with extraction) (Wintermantel, 2009). BNYVV is vectored by the plasmodiophorid, *Polymxa betae*, which is ubiquitous in soils (Rush,

2003). Because the vector is long-lasting in the soil, crop rotation is not a viable management strategy. Fumigation, which is expensive, and being phased out because of environmental concerns, has become a less available and less desirable management option. Therefore, genetic resistance in the form of resistant commercial hybrids is the preferred method of disease management (Panella and Biancardi, 2016; Pavli et al., 2011).

There are both foliar and root symptoms of BNYVV. Roots of infected plants have an abundance of lateral roots, which gives the root a "bearded" (rhizomania from the Greek for "crazy root") appearance, and the constriction of the taproot gives the root the so-called "wine glass" shape. Above ground, these symptoms and the blockage of the vascular tissue of infected plants causes wilting, which becomes more severe over time. Foliar symptoms also include a bright yellow coloring along the leaf veins, but foliar symptoms are not always observed and, indeed, a mild infection may not produce noticeable symptoms at all (Panella and Biancardi, 2016; Pavli et al., 2011; Wintermantel, 2009).
Although, at this time there are five *Bvm* rhizomania-resistant, single-gene sources described; the bulk of populations carrying known single gene sources from *Bvm* contain either *Rz1* or *Rz2* (Biancardi et al., 2002; Biancardi et al., 2012; Panella and Lewellen, 2007).

In locations where BNYVV has been able to overcome the resistance of *Rz1*, to provide sugar beet with tolerance to resistance-breaking-BNYVV (RB-BNYVV) strains, both *Rz1* and *Rz2* resistance genes are stacked in the commercial hybrids. Screening accessions from the USDA-ARS genebank using marker-aided selection would expedite the discovery of accessions with sources of BNYVV resistance and enhance the discovery of allelic variation.

Among molecular markers, single nucleotide polymorphisms (SNPs) present several advantages with respect to other genetic marker types (Schneider et al., 2001; Simko et al., 2012); they are anchored in the physical map and codominant. SNPs are abundant genetic markers available in sugar beet and have been used in mapping sugar beet crosses (Grimmer et al., 2007) and as markers for resistance genes (Stevanato et al., 2015; Stevanato et al., 2014; De Lucchi et al., 2017; Grimmer et al., 2007. The effective nematode-tolerant gene found in sea beet accession WB 242 (PI 546413), labeled *HsBvm-1,* was used to develop a SNP molecular marker (referred here as HsBvm-1) linked to nematode tolerance found in WB242 (Stevanato et al., 2014). A SNP marker (referred here as Rz1) linked to *Rz1* (Stevanato et al., 2012; Panella et al., 2015; Stevanato et al., 2014) was used to select individual families. Using marker assisted selection, a germplasm with a high frequency of resistance to sugar beet cyst nematode and rhizomania has been selected out of a single plant cross developed to verify the resistance to sugar beet cyst nematode conditioned by the *HsBvm-1* gene.

MATERIALS AND METHODS

Family Formation and Germplasm Development

A single plant cross was made between Salinas germplasm 9933 (PI 652891) (Panella et al., 2013) and an annual sea beet accession from the USDA-ARS National Plant Germplasm System's *Beta* collection, PI 546413, also called WB242. The purpose of this cross was to develop a population to map sugar beet cyst nematode (SBCN) tolerance because WB242 was considered a source of tolerance (Panella and Lewellen, 2007). WB 242 was collected from Loire river estuary in France and provided to J.S. McFarlane from a researcher at Bergen op Zoom, The Netherlands in May 1974 (GRIN, 2017). Germplasm 9933 (PI 652891) (Panella et al., 2013) is rhizomania resistant (segregates for Rz1 but does not have the Rz2 marker). This is a base breeding population, random mated and segregating for multigerm (MM), self-fertile (Sf), genetic-male-sterile (A:aa). In testing it has shown moderate to Aphanomyces root rot (caused by *Aphanomyces cochlioides*) and moderate to good resistance to Cercospora leaf spot (caused by *Cercospora beticola*). It also shows moderate resistance to powdery mildew (caused by *Erysiphe polygoni* [syn. *E. betae*]), curly top (caused by *Beet curly top virus*), and virus yellows (caused by *Beet chlorotic yellow virus*) (GRIN, 2017).

A single F_1 seed (labeled 20061023-05s) was selfed to produce seed of an F_2 family. Those seed were grown out in the greenhouse producing 125 plants, from which leaf tissue was collected for later DNA extraction and genotyping. Of the F_2 plants, 25 bolted without vernalization and were bagged to prevent cross-pollination. The remaining 100 plants were vernalized for 120 days and then allowed to put up flower stalks and were bagged in the greenhouse to prevent cross-pollination. These populations were the selfed seed of the 125 F_3 families from the original cross. Using the SNP markers Rz1 and HsBvm-1 (Stevanato et al., 2012; Stevanato et al., 2014) leaves from F_2 plants were genotyped for the markers Rz1 and HsBvm-1. Nine F_3 families were chosen to verify the greenhouse performance of the marker selection. These F_3 families were 20131045, 20131046, 20131047, 20131048, 20131049, 20131050, 20131051, 20131052, 20131053 (referred to further in the manuscript without the '2013' prefix).

There were 17 F_3 families, which were homozygous for markers Rz1 and HsBvm-1 selected and bulk increased in the greenhouse. There were 570 biennial plants which became seed production 20131058, and 62 annual plants which became 20131058B_. Seed production 20131058 was bulk increased in the greenhouse, and seed from 52 biennial plants became 20141038PF, the F_5 generation of this population. Seed from 20131058B_ also was bulked increased and seed from 93 biennial plants became 20141040. Seed of 20141038PF also was increased in the greenhouse as 20171002PF. Seed from 20171002PF had 100% green hypocotyls (*rr*) and was 21% monogerm. Weight of one hundred seeds

was 0.92 grams. Germination of 20171002PF was 166 sprouts per gram. This seed is released as FC242.

Experimental Procedures and Analysis

Because the HsBvm-1 marker was developed from a WB242 cross (Stevanato et al., 2014), we also were interested in verification of the marker in a greenhouse test. We knew the marker was present in commercial hybrids and accurately segregated with SBCN tolerance (Stevanato et al., 2014). We choose at random 3 F_3 families with the HsBvm-1 marker homozygous dominant (20131045, 20131046, 20131047 = NN), 3 families segregating for the HsBvm-1 marker (20131048, 20131049, 20131050 = Nn), and 3 families with HsBvm-1 marker homozygous recessive (20131051, 20131052, 20131053 = nn). Individual families were compared to Salinas control lines, N1012-446 (WB 242 source, moderately resistant), 10927-4-309 (moderately susceptible) and 10-C37 (very susceptible). Seed of these lines and the 9 F_3 families to be screened were planted in pasteurized sand to aid in germination. Two weeks after planting, two seedlings of each check line and 5 seedlings of each F_3 family were transplanted into Ray Leach Cone-tainers (66 cm3; Stuewe and Sons, Tangent, OR) containing soil naturally infested with SBCN at approximately 20 larvae cm–3 soil. The experiment was a randomized complete block design with subsampling. The experiment is blocked with 6 planting dates and five seedlings at each date as blocks. Each replicate was a rack of 56 Cone-tainers with 1 seedling in each. Sugar beet seedlings were removed from the Cone-tainers 56 d after transplanting, and the roots were rinsed with water to remove soil and poured over sieves (#20 and #60). The cysts were collected and washed into a sample container. The cyst solution was poured into a watch glass, and the number of cysts was counted under a dissecting microscope.

Cyst counts on individual seedlings of the three lines were analyzed with SAS software version 9.4. The GLIMMIX procedure was used to fit the observed cyst counts to a generalized linear mixed model with cyst counts modeled as negative binomial with a log link. Lines were modeled as fixed effects and racks and racks crossed with lines were modeled as random effects. Estimates of the mean cyst counts for the treatments were obtained from the fitted model. Contrasts of means were performed to make specific comparisons among the three categories of F_3 families. A Dunnett's one-tailed t-test with a Dunnett-Hsu adjustment for multiple comparisons was used to compare the model means of each family and check to the very susceptible check and to the moderately resistant check. The F_3 families were compared in groups to further examine 1048, which performed differently from the other segregating families. It was compared to the other two segregating families. The other two segregating families (without 1048) and all three segregating families were compared the homozygous resistant and homozygous susceptible families. Because the family, 1048, performed phenotypically

different from the other two segregating families, 96 remnant seeds from the F_3 family was genotyped using the HsBvm-1 marker. A chi square test was performed to see if the segregation ratio conformed to the expectation of a family from a selfed, heterozygous F_2-plant

RESULTS

Individual families were compared to each other and check lines using a Dunnett's one-tailed t-test (P=0.05) to either the very susceptible check or the moderately resistant check (Table 1). The moderately susceptible and very susceptible checks were not significantly different, but the very susceptible check and the moderately resistant check were significantly different. The families homozygous for the tolerance marker (1045, 1046, and 1047) were not significantly less resistant than the moderately resistant check and were significantly more resistant than the very susceptible check (Table 1). Those families homozygous for susceptibility based on the marker (1051, 1052, and 1053) were not significantly more resistant than the very susceptible check and were significantly more susceptible than the moderately resistant check (Table 1). It can be seen that 1048 performed differently than the other two segregating families (1049 and 1050). With the one tailed t-tests, 1048 was not significantly more resistant than the very susceptible check line (P = 0.06) and it was significantly more susceptible than the moderately resistant check (Table 1).

To more closely examine differences among the F_3 families (Figure 1), contrasts were constructed and tested for significance (P = 0.05) (Table 2). There were significant differences between the homozygous tolerant (NN) and homozygous susceptible families (nn) (Table 2). All three families segregating families (Nn) were compared to the three susceptible families and found to be significantly different as they were from the three tolerant families (Table 2). When the two segregating families (1049 and 1050) were compared to the 3 tolerant families (NN), they were significantly more susceptible and when they were compared with the 3 susceptible families (nn) they were significantly more resistant (Table 2). 1048 was compared to 1049 and 1050 (all three segregating in the F_2 for the marker – Nn) and was found to be significantly different (Table 2. This family (1048) was not significantly different from the very susceptible check and was significantly different from the moderately resistant check when it was compared with the check lines in the one-tailed t-test (Table 1). Remnant seed of 1048 was genotyped and the marker segregated in a ratio of 18:13:65 (NN:Nn:nn). The expected genotypic ratio for an F_3 family of a selfed, heterozygous F_2-plant would be 1:2:1. A chi square tests showed that the F_3 ratio of plants' genotypes in the 1048 family is significantly different than the expectation (Table 3).

Table 1. Differences of Treatment Least Squares Means - Adjustment for Multiple Comparisons: Dunnett-Hsu. The GLIMMIX procedure was run and least square means generated on cyst counts on individual seedlings of control plants and plants whose performance was predicted by a SNP marker linked to the HsBvm-1 resistance gene. A Dunnett's one-tailed t-test with a Dunnett-Hsu adjustment for multiple comparisons was used to compare the number of cysts on each line (treatment) and check to the number of cysts on the very susceptible check and the moderately resistant check.

Treatment	Treatment	Estimate	Standard Error	DF	T Value	Pr < t	Adj P
Very Susceptible	Moderately Resistant	1.7336	0.3195	60	5.43	<.0001	<.0001
Very Susceptible	Moderately Susceptible	0.5623	0.3130	60	1.80	0.0387	0.1782
Very Susceptible	1048 (Nn)	0.6516	0.2737	60	2.38	0.0102	0.0601
Very Susceptible	1047 (NN)	1.6491	0.2712	60	6.08	<.0001	<.0001
Very Susceptible	1050 (Nn)	1.3749	0.2712	60	5.07	<.0001	<.0001
Very Susceptible	1049 (Nn)	1.3330	0.2700	60	4.94	<.0001	<.0001
Very Susceptible	1053 (nn)	0.2217	0.2694	60	0.82	0.2069	0.5701
Very Susceptible	1046 (NN)	1.7634	0.2736	60	6.45	<.0001	<.0001
Very Susceptible	1051 (nn)	0.4833	0.2694	60	1.79	0.0389	0.1790
Very Susceptible	1045 (NN)	2.0846	0.2708	60	7.70	<.0001	<.0001
Very Susceptible	1052 (nn)	0.1069	0.2698	60	0.40	0.3466	0.7484
Moderately Susceptible	Moderately Resistant	1.1713	0.3133	60	3.74	0.0002	0.0018
1048 (Nn)	Moderately Resistant	1.0820	0.2694	60	4.02	<.0001	0.0007
1047 (NN)	Moderately Resistant	0.0845	0.2717	60	0.31	0.3785	0.7791
1050 (Nn)	Moderately Resistant	0.3587	0.2692	60	1.33	0.0939	0.3432
1049 (Nn)	Moderately Resistant	0.4006	0.2710	60	1.48	0.0723	0.2850
1053 (nn)	Moderately Resistant	1.5119	0.2708	60	5.58	<.0001	<.0001
1046 (NN)	Moderately Resistant	-0.0298	0.2723	60	-0.11	0.5434	0.8958
1051 (nn)	Moderately Resistant	1.2503	0.2711	60	4.61	<.0001	0.0001
1045 (NN)	Moderately Resistant	-0.3510	0.2703	60	-1.30	0.9005	0.9957
1052 (nn)	Moderately Resistant	1.6267	0.2704	60	6.02	<.0001	<.0001
Very Susceptible	Moderately Resistant	1.7336	0.3195	60	5.43	<.0001	<.0001

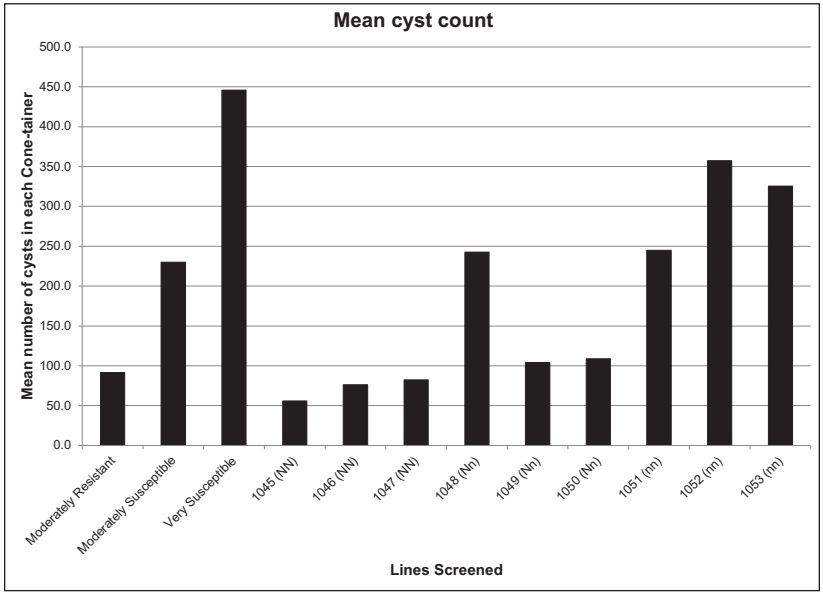
Table 2. The GLIMMIX procedure was run and least square means generated on cyst counts on individual seedlings. The F_3 families (1045 through 1053) were assigned a genotype based on the HsBvm-1 SNP marker linked to the *HsBvm-1* gene. The different sets of the F_3 families were contrasted – Marker selected homozygous resistant (NN), homozygous susceptible (nn), and segregating (Nn).

Contrasts			Num DF	Den DF	F Value	Pr > F
Nn (1048, 1049,1050)	vs	**NN** (1045, 1046, 1047)	1	60	32.90	<.0001
Nn (1048, 1049,1050)	vs	**nn** (1051, 1052, 1053)	1	60	47.19	<.0001
Nn (1049, 1050)	vs	**NN** (1045, 1046, 1047)	1	60	12.05	0.0010
Nn (1049, 1050)	vs	**nn** (1051, 1052, 1053)	1	60	62.71	<.0001
NN (1045, 1046, 1047)	vs	**nn** (1051, 1052, 1053)	1	60	161.00	<.0001
Nn (1048)	vs	**Nn** (1050, 1049)	1	60	14.23	0.0004

Table 3. Remnant seed of F_3 family 1048 was genotyped with the *HsBvm-1* marker linked to sugar beet cyst nematode resistance. The F_2 plant had been genotyped with the marker as heterozygous, and, therefore, the expected ratio in the F_3 is 1:2:1. When the observed ratio is compared to the expected ratio, the X^2 statistic is 39.8268 and the probability that the observed ratio is the same as the expected is $p<0.00001$.

Ratio	Homozygous Resistant (NN)	Heterozygous (Nn)	Homozygous Susceptible (nn)
Observed	18	13	65
Expected	24	48	24

Figure 1. Seed of 9 F_3 families and 3 control germplasm populations were screened in soil naturally infested with sugar beet cyst nematode (SBCN). Two weeks after planting, two seedlings of each control population (3 germplasms) and 5 seedlings of each F_3 family (9 families) were transplanted into Cone-tainers (one individual plant in each Cone-tainer), and after 56 days SBCN cysts were counted from each individual Cone-tainer (plant). Mean number of cyst per family and control populations are shown below.



DISCUSSION

WB242 has been used as a source of tolerance for SBCN in the USDA-ARS breeding program (Lewellen and Pakish, 2005; Lewellen, 2006; Lewellen, 1995; Lewellen, 2007; reviewed by Panella and Lewellen, 2007; Richardson, 2012). Additionally, WB242 has been a well-used and well-researched resource for bringing other disease resistances into the cultivated beet genepool. Powdery mildew, introduced into the United States sometime before 1974, produced an epidemic in 1974, with losses as great as 30% (Ruppel et al., 1975). It was most often managed with chemical protectants. CP02 and CP04, derived from WB242, were released with resistance to powdery mildew (Lewellen, 2000, 2004), which was then shown to be conditioned by a single gene, *Pm* (Lewellen and Schrandt, 2001; Janssen et al., 2003). WB242 also was a component of CP07, which had been selected under natural powdery mildew, rhizomania, and cyst nematode infested conditions for resistance to these diseases and pest (Lewellen, 2004).

The examination of F_3 families, which had an inferred genotype based on the HsBvm-1 marker, was useful. Those families chosen as homozygous tolerant or homozygous susceptible performed as expected when compared to tolerant and susceptible check lines. More interesting were those families in which the marker was segregating in the selfed F_2 plant. One family (1048) did not perform significantly different from the susceptible checks in the one-tailed t-test (Table 1), and when contrasted with the other two segregating families was significantly different from them (Table 2). The other two segregating families (1049 and 1050) performed as expected – intermediate between the tolerant and susceptible families. We know that many of the tolerant commercial hybrids contain only one copy of the marker and provide enough SBCN control to be economically viable. A chi square test showed that this F_3 family (1048) had a skewed segregation ratio, which is not unknown in such crosses (personal communication, Mitch McGrath (2017)). We expected the HsBvm-1 marker to segregate in a 1:2:1 (NN:Nn:nn) manner and our marker showed that there was a problem in segregation and, in a breeding program we would have been able to reselect. This marker has been useful in a pre-breeding program and allows an increased efficiency by permitting selecting a population of enhanced frequency of the *HsBvm-1* gene before the line is taken for field testing.

Seed of this population will be maintained by USDA-ARS, Fort Collins, Colorado and freely distributed in quantities sufficient for reproduction. Requests for seed should be directed to USDA-ARS-CARR, Soil Management and Sugar Beet Research Unit, 1701 Centre Ave, Fort Collins, CO 80526. Seed samples also have been deposited with the USDA National Plant Germplasm System where they will be available for research purposes, including the development of new varieties. Plant Variety Protection will not be pursued for this population.

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