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ORIGINAL PAPER

Identification and Validation of a SNP Marker Linked to the Gene *HsBvm-1* for Nematode Resistance in Sugar Beet

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Abstract The beet-cyst nematode (*Heterodera schachtii* Schmidt) is one of the major pests of sugar beet. The identification of molecular markers associated with nematode tolerance would be helpful for developing tolerant varieties. The aim of this study was to identify single nucleotide polymorphism (SNP) markers linked to nematode tolerance from the *Beta vulgaris* ssp. *maritima* source WB242. A WB242-derived F_2 population was phenotyped for host-plant nematode reaction revealing a 3:1 segregation ratio of the tolerant and susceptible phenotypes and suggesting the action of a gene designated as *HsBvm-1*. Bulked segregant analysis (BSA) was used. The most tolerant and susceptible individuals were pooled and subjected to restriction site associated DNA sequencing (RAD-Seq) analysis, which identified 7,241 SNPs. A subset of 384 candidate SNPs segregating between bulks were genotyped on the 20 most-tolerant and most-susceptible individuals, identifying a single marker (SNP192) showing complete association with nematode tolerance. Segregation of SNP192 confirmed the inheritance of tolerance by a single gene. This association was further validated on a set of 26 commercial tolerant and susceptible varieties, showing the presence of the SNP192 WB242-type allele only in the tolerant varieties. We identified and mapped on chromosome 5 the first nematode tolerance gene (*HsBvm-1*) from *Beta vulgaris* ssp. *maritima* and released information on SNP192, a linked marker valuable for high-throughput, marker-assisted breeding of nematode tolerance in sugar beet.

Keywords *HsBvm-1* · *Beta vulgaris* ssp. *maritima* · Biotic stresses · Beet-cyst nematode · *WB242* genetic tolerance · SNP

Abbreviations

SNP	Single nucleotide polymorphisms
RAD-Seq	Restriction site associated DNA sequencing
BSA	Bulk segregant analysis

Introduction

Sugar beet (*Beta vulgaris* L.) provides about a third of all sugar consumed worldwide (Biancardi et al. 2010). The crop is damaged by many different diseases and the identification of molecular markers associated with disease resistance would be helpful for developing resistant varieties. Among molecular markers, single nucleotide polymorphisms (SNPs) present several advantages with respect to other genetic marker types. SNPs are the most abundant genetic markers available in sugar beet and a wide array of technologies have been developed to very quickly genotype large numbers of SNPs in DNA samples (Stevanato et al. 2013). The development of a large set of SNP markers could facilitate the identification and exploitation of genes affecting important traits. Several

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techniques are used to enable SNP marker discovery in plants. Among them, the "restriction site associated DNA" (RAD) technique is widely used (Miller et al. 2007). The RAD technique is based on acquiring and characterizing the genomic regions adjacent to a set of specific restriction enzyme recognition sites (Davey et al. 2011). The bulk segregant analysis (BSA) is a method for identifying DNA markers linked to genes or genomic regions of interest (Michelmore et al. 1991). DNA samples from individuals showing contrasting phenotype are compared with a large set of molecular markers to identify those linked to the trait of interest. Among sugar beet diseases, a major constraint to production is beet-cyst nematode (*Heterodera schachtii* Schmidt). The disease is spread over 40 sugar beet growing countries (McCarter 2008). It causes yield losses up to 60 % and typical symptoms are massive proliferation of secondary roots and the weak development of the beets (Biancardi et al. 2010). Management of nematodes is becoming harder because nematicides are no longer available (Thurau et al. 2010). Also, wide crop rotations with nonhost plants (e.g., wheat, barley, corn, beans, and alfalfa) often are not economically practical (Kleine et al. 1998). In this context, the introduction of nematode tolerance into sugar beet is an efficient management measure available for nematodes (Jung et al. 1998). Numerous nematode resistance genes have been identified from plants that exhibit resistance against nematodes. In sugar beet, the first cloned nematode resistance gene *Hs1* gene has been introduced from the wild species *Patellifolia procumbens* (Cai et al. 1997). An effective nematode-tolerant source was also found in sea beet (*B. vulgaris* subspecies *maritima* (L.) Arcang.) accession WB242, collected at Loire River Estuary in France (Biancardi et al. 2012). The aim of this study was the development of SNP molecular markers linked to nematode tolerance found in WB242. To achieve this aim, a BSA strategy combining advanced DNA technologies (RAD-sequencing and high-throughput SNP genotyping) was used.

Material and Methods

Plant Material

To identify SNP markers linked to the nematode tolerance of the *B. vulgaris* ssp. *maritima* source, WB242, a segregating F_2 population, was developed by crossing the tolerant pollinator (WB242) with a nematode-susceptible male sterile line (CMS_1). Seeds of the WB242 line were obtained from the USDA-ARS, NPA, Sugarbeet Research Unit, Crops Research Laboratory at Fort Collins (USA) and seeds of the CMS_1 line were provided by CRA-Research Institute for Industrial Crops (Rovigo, Italy). Seeds derived from individual F_1 and F_2 plants were produced during 2011 and 2012, respectively, at the University of Padova (Italy). In addition to the F_2 population, a set of 13 tolerant and 13 susceptible commercial varieties, provided by BETA SCARL (Ferrara, Italy), were used to further examine the association between phenotypic tolerance and markers identified in this study. Greenhouse trials carried out in the period of 2005 to 2009 by BETA SCARL showed that the number of cysts detected in the tolerant varieties, under nematode infection, was averaging 50 % lower than that in the susceptible varieties.

Phenotyping Analysis

A total of 384 F_2 plants were grown in the greenhouse of the USDA-ARS, Crop Improvement and Protection Research Unit at Salinas, CA (USA). F_2 seeds were germinated in pasteurized sand and transplanted into Ray Leach Cone-tainers (Stuewe & Sons, Inc., USA) filled with naturally nematode-infested soil adjusted to 20 cysts per gram. Seventy-day-old seedlings were removed from cones and roots were rinsed with water over sieves to remove soil. Cysts were collected and washed into a sample container. The cyst solution was poured into a watch glass and the number of cysts in the soil was counted under a dissecting microscope to assess the level of nematode tolerance. The tolerant (WB242) and susceptible (CMS_1) parental lines also were included in the analysis as internal controls.

DNA Isolation

DNA was isolated with the BioSprint 96 DNA Plant Kit (QIAGEN, Germany) in a BioSprint 96 workstation (QIAGEN, Germany) following the manufacturer's instructions. Leaf samples were ground using a QIAGEN TissueLyser (QIAGEN). Briefly, 20 mg of leaf tissue were placed into 2-ml tubes and 300 μl of RLT buffer (guanidine thiocyanate buffer under patent protection) were added to each sample. One stainless steel 5-mm bead was used for every sample, which was then homogenized for 10 min at 30 Hz. Samples were centrifuged at 6,000 g for 5 min and supernatant loaded into a 96-well plate with 200 μl of isopropanol and 20 μl of magnetic beads suspension. The beads were transferred consecutively into four other plates each with a premix, followed by a 4-min binding step and one bead collection step. The first plate was loaded with RPW buffer (guanidine thiocyanate buffer under patent protection). The second and third plates were loaded with 500 μl of 96 % ethanol. The fourth plate was loaded with 500 μl of 0.02 % (*v*/*v*) of Tween 20. DNA was eluted with 200 μl of sterile Milli-Q water. After isolation, DNA was assayed for concentration and purity by microfluidic gel electrophoresis with the Agilent 2200 TapeStation system (Agilent Technologies, CA, USA). The average DNA yield was 50 ng μl^{-1} with an average 260:280 ratio of 1.85.

Linked-SNP Discovery by RAD-BSA

Based on the F_2 nematode tolerance analysis, normalized DNAs of the four most tolerant (T) and susceptible (S) samples were pooled for bulked segregant analysis (BSA) to form the tolerant and susceptible bulks, respectively. Samples were sent to Floragenex (Oregon, USA), which carried out the restriction-associated DNA (RAD) analysis following the methods outlined by Pegadaraju et al. (2013). Initially, 2× 60 bp sequence data produced from an Illumina Genome Analyzer II was sorted by the appropriate multiplex index (MID) or "barcode" assigned to each sample during RAD-Seq library construction. Reads from the T samples were selected for RAD paired end sequence assembly. First, reads were trimmed to remove low-quality sequences with an average Phred-scaled quality score below 25 (Q25) at the 3′ end of reads. Reads passing these filters were then collapsed into RAD sequence clusters sharing 100 % sequence identity across the first 50 bp of the single -end Illumina read. To maximize efficient assembly of sequences, we imposed a minimum of 20× and a maximum of 1,000× sequence coverage at any RAD sequence cluster. The paired end sequences meeting these criteria were extracted for each RAD cluster and then passed to the Velvet sequence assembler for contig assembly. Sequence reads from S samples were then aligned to reference assembly for T samples using Bowtie. Alignment thresholds were specified which allowed up to three base pair mismatches between the Illumina read and the reference and only unique alignments between query and reference were considered. Putative sequence variants from the alignments were then called using SAMtools. To be considered for genotyping design, a SNP had to have a minimum Phred-scaled genotype quality of 15 across each of the 3 samples, with at least 50 bp of flanking genomic sequence surrounding the target SNP. Variants with nearby flanking polymorphisms within 50 bp of the candidate marker were also excluded from further consideration for genotyping design. Additionally, contigs assembled from T samples containing sequence polymorphisms meeting the criteria above were aligned to the sugar beet reference genome (version RefBeet-0.9; http://bvseq.molgen.mpg.de), allowing for a maximum of a single mismatch to provide a genomic anchor and location for the newly discovered SNP.

Linked-SNP Validation by Genotyping

From the SNP discovery analysis, a total of 384 candidate SNPs were selected for validation. Genotyping was performed on the 20 most tolerant and most susceptible single F_2 individuals. SNPs were screened using the QuantStudio 12 K Flex real-time PCR system and OpenArray technology (Life Technologies, CA, USA). A total of 10 ng of DNA sample was mixed with 2.5 μl of TaqMan OpenArray Genotyping Master Mix in a 384-well plate. Samples were subsequently loaded onto the OpenArray plate using the QuantStudio 12 K FlexOpenArray AccuFill System. Following PCR, allelic discrimination results were analyzed using the Taqman Genotyper software (ver.1.0.1).

Statistical and Linkage Analysis

Frequency distribution of the F_2 population was tested for normality using the Shapiro-Wilk tests (Conover, 1980). A χ^2 test was used to compare observed and expected ratios in the F_2 generation. Combining phenotypic and genotypic data, 384 SNP markers were genotyped on 384 F_2 individuals to construct a genetic map. JoinMap® version 4.1 was used for linkage analysis and map calculations. Marker order and genetic distance were calculated using the Kosambi mapping function (Van Ooijen 2011). The critical thresholds adopted for the analysis was a LOD score of 5.0.

Results

Nematode Tolerance Analysis

The frequency distribution of number of nematode cysts in the F_2 progeny and in the parental lines is shown in Fig. 1. The mean number of cysts present in the tolerant (WB_242) and susceptible (CMS_1) controls were 22 and 130, respectively, while the distribution of cyst counts among the 384 F_2 individuals ranged from 0 to 159 and was distributed according to the normal distribution (Shapiro-Wilk's, test; $P<0.05$).

Linked-SNP Discovery by RAD-BSA

From the RAD sequencing analysis, a total of 98,975,012 raw reads was obtained from the two bulks, of which 82,031,123 were of high quality (82.7 %). These reads were aligned and yielded a total of 266,723 unique consensus RAD-tags common between bulks, with an average of 150× coverage per bulk sample. The SNP discovery pipeline identified a total of 7,241 high-quality SNPs, of which 384, mainly polymorphic between bulks, were selected for further analysis as markers putatively linked with nematode tolerance loci.

Linked-SNP Validation by Genotyping

Validation of the 384 putative SNP selected from the RAD-BSA analysis was performed by individually genotyping the 20 most-tolerant and most-susceptible F_2 samples. By comparing SNP genotyping data, a single marker (SNP192) showed complete association with nematode tolerance for all individuals analyzed, whereas no significant association was

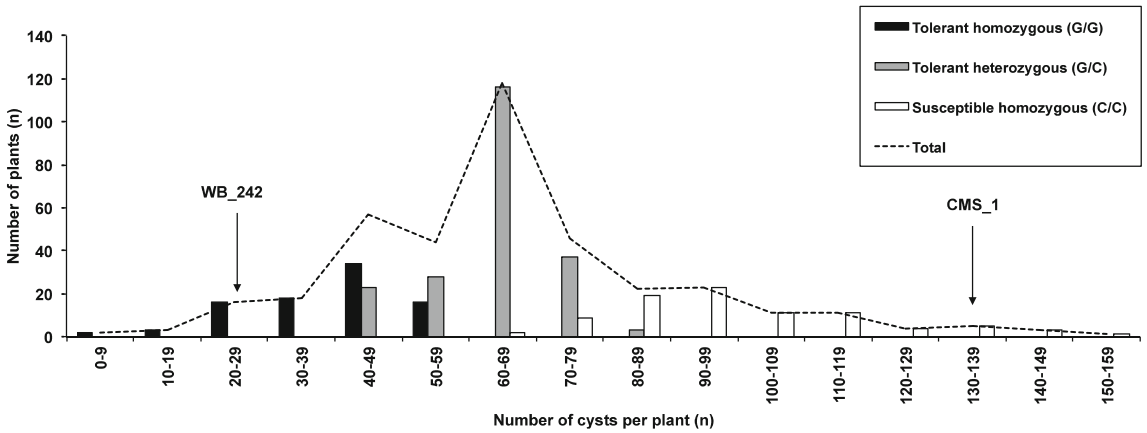

Fig. 1 Frequency distribution of number of nematode cysts in the F_2 population (*dashed line*) and allele frequencies of the linked marker SNP192 (*histograms*)

found for the other SNPs. SNP192, found on scaffold00252 of the RefBeet-0.9 reference genome, was mapped on chromosome 5 (Fig. 2).

Scoring all the F_2 individuals with the SNP192, identified in this study, showed tolerant homozygous (G/G) individuals with an average of 39 cysts/plant, tolerant heterozygous (G/C) individuals with an average of 61 cysts/plant, and susceptible homozygous (C/C) with an average of 101 cysts/plant. Moreover, the genotyping analysis of the candidate-linked marker SNP192 confirmed that the resulting ratio of segregation was consistent with that of a dose-effect single gene (Table 1). The SNP192 association pattern was confirmed further on a set of 260 individuals representing 26 genotypes (10 individuals each) from 13 tolerant and 13 susceptible commercial varieties. In all tested individuals, a complete association of the marker with phenotypic tolerance was observed, with tolerant and susceptible varieties being heterozygous (G/C) and homozygous (C/C), respectively (Table 2).

The two alleles of the SNP192 and its flanking sequences on each side of the SNP are reported as supplementary material (Table S1). Also, the sequences of the primers and TaqMan probes designed for the detection of the SNP192 are available as supplementary material (Table S2).

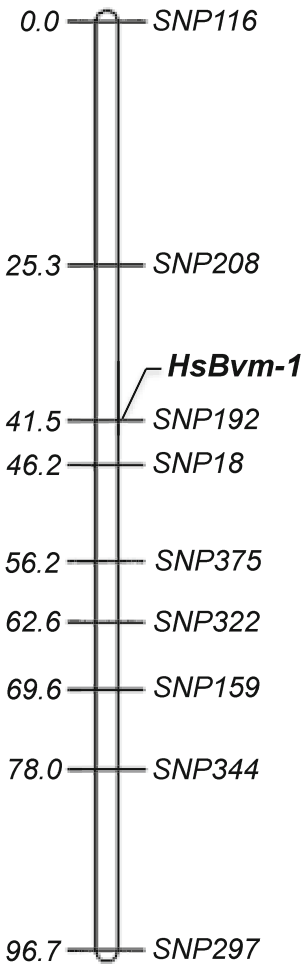

Fig. 2 Genetic map of SNP markers on chromosome 5 and location of the WB242 gene. Distances are shown in centiMorgans (cM)

Discussion

This study was designed to identify SNP markers linked to nematode tolerance by means of BSA analysis. This aim was achieved through the following main steps: (i) the phenotyping analysis of 384 F_2 individuals for nematode tolerance, (ii) the RAD-sequencing of DNAs of tolerant and susceptible

Table 1 Segregation analysis for observed ratios of the SNP192 alleles in the F_2 population

Marker	Observed ratios			χ^2
	G/G	G/C	CC	
SNP192	89	210	85	0.901 ns

ns not significant

Table 2 SNP192 alleles in tolerant and susceptible sugar beet varieties

Name	Seed company	Phenotype	SNP192 alleles
Massima	KWS	Tolerant to nematode	G/C
Kepler	Strube	″	G/C
Eclipse	Betaseed	″	G/C
Atleta	Syngenta	″	G/C
Baobab	SesVanDerHave	″	G/C
Bison	SesVanDerHave	″	G/C
Bramata	Syngenta	″	G/C
Bruna	KWS	″	G/C
Cactus	SesVanDerHave	″	G/C
Charly	Strube	″	G/C
Ciclon	SesVanDerHave	″	G/C
Dallas	Betaseed	″	G/C
Karta	Syngenta	″	G/C
Tarim	Desprez	Susceptible to nematode	C/C
Rosalinda	KWS	″	C/C
Boogie	Maribo	″	C/C
Sarbacane	SesVanDerHave	″	C/C
Cetus	Strube	″	C/C
SY Invicta	Syngenta	″	C/C
Bandit	Syngenta	″	C/C
Britta	KWS	″	C/C
Magistral	SesVanDerHave	″	C/C
Roberta	KWS	″	C/C
Dorotea	Syngenta	″	C/C
Perfekta	Lion Seeds	″	C/C
Aaron	Lion Seeds	″	C/C

plants, and (iii) the high-throughput SNP genotyping of the 20 most-tolerant and the 20 most-susceptible individuals with newly discovered candidate SNPs.

Phenotype Analysis

The phenotype analysis revealed that the segregation ratio of the number of the cysts in the population supported that nematode tolerance was controlled by a single gene. The gene was designated here as *HsBvm-1* being the first gene for tolerance to *H. schachtii* from (*B. vulgaris* L. *maritima*. Also, it is the first gene for tolerance to nematode mapped on chromosome 5. Other monogenic sources of resistance to nematodes have been found in *P. procumbens* and *Patellifolia webbiana*: *Hs1* on the homologous chromosomes 1 of each species, *Hs2* on the homologous chromosomes 7 of *P. procumbens* and *P. webbiana* and *Hs3* on chromosome 8 of *P. webbiana* (Thurau et al. 2010). The transfer of the beet cyst nematode resistance from *Patellifolia* sp. to cultivated beet was made by species hybridization (Panella and Lewellen 2007), although the transmission rate was very low due to meiotic disturbances (Brandes et al. 1987). In sugar beet, some other important disease resistance traits are inherited as single genes. The "Rizor"-type resistance to rhizomania was recognized as monogenic and dominant (Biancardi et al. 2002). Also the resistance to *Aphanomyces* was designated as monogenic and dominant (Taguchi et al. 2010).

RAD-SNP Discovering

The phenotyping analysis allowed the identification of two tolerant and susceptible groups that were subsequently submitted to RAD-sequencing. This technique was efficiently used to identify over 7,000 SNPs with the aim of development of an appropriate panel of SNP markers for the BSA analysis. Analogously, this approach allowed the identification of more than 10,000 SNPs to fingerprint different eggplant genotypes (Barchi et al. 2011). In barley, RAD technique was applied to construct a linkage map and to detect SNPs linked to QTLs for reproductive traits (Chutimanitsakun et al. 2011).

Bulked Segregant Analysis

The bulked segregant analysis (BSA) analysis allowed the identification of a SNP linked to nematode tolerance that can be used in the breeding programs. Additionally, BSA has been successfully used in sugar beet for identifying markers linked to important traits of interest such as rhizomania (Pelsy and Merdinoglu 1996), male sterility (Touzet et al. 2004) and root elongation rate (Stevanato et al. 2010). The mapping of this SNP marker on the sugar beet reference genome (version RefBeet-0.9) allowed the precise localization of the *HsBvm-1* locus. Finally, SNP192 showed a complete association with the phenotypic tolerance in a total of 384 genotyped F_2 individuals. The genotyping of commercial tolerant and susceptible varieties with SNP192 confirmed its association with nematode tolerance. All individuals from the tolerant varieties showed the SNP192 allele corresponding to the tolerant heterozygote allelic status, suggesting that they shared the same tolerance source *HsBvm-1* from sea beet accession WB242. An analogous assumption has been suggested for the monogenic resistances to rhizomania derived from sea beet (Holly and WB42), which are present in the current resistant varieties (Biancardi et al. 2002).

Conclusion

A SNP marker (SNP192) showing a complete association to the nematode tolerance gene *HsBvm-1* was identified by the successful use of the BSA approach. As previously seen, this study revealed that sea beet is an invaluable source of resistances for sugar beet breeding. The SNP192 and the related

TaqMan discrimination assay are recommended for high-throughput marker-assisted breeding of nematode tolerance in sugar beet. The use of this molecular marker linked to nematode tolerance is advantageous with respect to conventional selection, which requires time-consuming steps and higher breeding costs.

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