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Phenotypic and molecular characterization of the response to two soil-borne viruses in durum wheat landraces for the identification of new sources of resistance

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ABSTRACT

Durum wheat cultivation is increasingly threatened by viral diseases worldwide. Soil-borne cereal mosaic virus (SBCMV) and wheat spindle streak mosaic virus (WSSMV) cause significant crop losses in Europe. These viruses are transmitted through a soil-inhabiting vector, the plasmodiophoromycota *Polymyxa graminis* Led. There are very few methods available to eradicate *P. graminis*, whose resting spores survive in infested soil for decades, but they are either too expensive or not environmentally friendly. Therefore, it is crucial to develop resistant wheat varieties to mitigate the damage. For this purpose, more than 200 durum wheat genotypes, mostly landraces, were selected from the Global Durum Wheat Panel germplasm collection. Then, an experiment was conducted in a semi-controlled environment: the genotypes were sown in pots containing soil infested by *P. graminis* carrying SBCMV and WSSMV and maintained through the winter period. In early spring, visual assessment of viral symptomatology was performed. Subsequently, the viral loads of the two viruses in leaf tissues were determined through qRT-PCR analysis. The tested genotypes exhibited different responses to the two viruses: SBCMV showed very diversified viral loads among genotypes, whereas WSSMV infected all genotypes. We identified 23 genotypes, with low viral loads of both viruses and reduced symptoms, that could be of particular interest for breeders aiming at new resistant durum wheat varieties. A pilot GWAS allowed to identify genomic regions putatively associated to resistance to SBCMV or WSSMV, as well as possible candidate genes involved in these traits.

1. Introduction

According to the Food and Agriculture Organization (FAO), in 2022, the global area dedicated to wheat (common and durum wheat) cultivation exceeded 219 million hectares, which represents approximately 30 % of the total area dedicated to cereal cultivation. It has been reported that the global wheat harvest has yielded over 808 million tons (https://www.fao.org/faostat/en, accessed on 15/03/2024). It is worth noting that wheat is the most used cereal for food purposes and is considered one of the most important cereal crops due to its chemical composition and unique technological properties [1]. Pasta is a widely enjoyed food around the world, and it can be found in both fresh and dried forms. Durum wheat (*Triticum turgidum* L. subsp. *durum* (Desf.) Husnot) is commonly used as the raw material for dry pasta due to its protein rheology and pigment content. In certain countries such as Italy, France, and Greece, national laws require the use of durum wheat semolina as the sole raw material for pasta production [2].

Several plant pathogens, including viruses, are known to affect this cereal. Soil-borne wheat viruses have been reported worldwide [3], and their spread and severity are increasing due to climate change [4]. They are transmitted by the plasmodiophorid *Polymyxa graminis* Led. and can persist in soil for up to 20 years [5–7]. A number of soil-borne viruses

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have been described in wheat and other cereals and two are relevant in European countries: the furovirus *soil-borne cereal mosaic virus* (SBCMV) and the bymovirus *wheat spindle streak mosaic virus* (WSSMV). In addition, the *soil-borne wheat mosaic virus* (SBWMV), another furovirus that is very close to SBCMV and widespread in U.S.A. has been reported in Germany [8]. These viruses have elongated particles and bipartite ssRNA genomes [5]. It is worth noting that these viruses affect both durum and common wheat (*Triticum aestivum* L.), as well as the hybrid cereal triticale and rye (*Secale cereale*) [3]. Wheat fields with yellow mosaic symptoms frequently have mixed infections of soil-borne viruses, as observed in Italy, France, USA, and Zambia [9–12]. Co-infections in fields can cause a significant reduction of up to 70 % in wheat production [13,14]. Growing resistant cultivars is the only solution to ensure the cultivation of wheat in infested areas, as chemical means for controlling *P. graminis* are either ineffective or unacceptable for ecological and economic reasons [15].

Although most of research on soil-borne virus resistance has been conducted on common wheat, a few reports tested durum wheat reaction to SBCMV, WSSMV, or their combination in field conditions [12–14, 16–19]. Kuhne and co-workers suggested that there is no apparent correlation between wheat resistances to SBCMV and WSSMV [3]. In these works, the assessments were based on visual analysis of symptoms and on enzyme-linked immunosorbent assay (ELISA) tests; however, visual analysis can easily be confused with damage caused by physiological stresses.

In common wheat, SBCMV resistance is mainly controlled by a single locus, *Sbm1*, mapped on the distal end of the long arm of chromosome 5D, not present in the durum wheat genome [20–22]. Further research led to the discovery of another locus called *Sbm2* on the short arm of chromosome 2B in bread wheat that controls resistance to SBCMV. It has been demonstrated that this locus acts distinctly and complementarily to *Sbm1* [23]. Maccaferri and colleagues conducted an in-depth investigation in durum wheat using a population of recombinant inbred lines (RILs) derived from Italian cultivars [24,25]. During the field testing, it was observed that the variability in SBCMV response was mostly attributed to a major QTL, *QSbm.ubo-2B*, which is located in the distal telomeric region of chromosome 2BS coincident with *Sbm2*, previously mapped in hexaploid wheat. This QTL accounts for a significant proportion (60–70 %) of the phenotypic variation. Furthermore, minor QTLs that affect the response to SBCMV infection were also identified. A new genetic locus, named *Sbm3*, was recently identified on the A genome in *Triticum monococcum* and successfully transferred to hexaploid wheat [26].

Studies on WSSMV resistance in common wheat identified a locus on the long arm of chromosome 2D, not present in durum wheat [27]. For several years, studies have focused on the development of wheat--*Haynaldia villosa* translocation lines. The WSSMV resistance gene named *Wss1* was transferred by a translocation from the short arm of the 4 V chromosome of *H. villosa* to the long arm of the 4D chromosome of wheat [28–30]. Finally, two major QTLs located on chromosomes 7 A and 7B and one minor QTL on chromosome 2 A were also identified to be associated with WSSMV resistance [31].

The identification of new loci associated with resistance to soil-borne viruses could be very useful for breeding programs. Due to the limited knowledge about resistant wheat genotypes, combined phenotyping and high throughput genotyping approaches through association analysis will allow the identification of potential further QTLs for resistance. Genome wide association studies (GWAS) are powerful tools to identify genomic regions and related candidate genes involved in virus resistance and offer the opportunity to accelerate mapping of both monogenic and polygenic resistance [32]. Recently, a GWAS was performed to find genomic regions associated to SBWMV resistance and two regions on chromosomes 5DL and 4DS of bread wheat were found [33,34]. More recently, this approach was used on durum wheat to dissect the genetic basis of SBCMV resistance related to the QTL *QSbm.ubo-2B* [35]. Regarding WSSMV, Hourcade and colleagues identified through GWAS two regions on bread wheat chromosomes 5B and 7D with minor effects on WSSMV resistance and a large genomic region on chromosome 2D that explained most of the resistance to WSSMV [36]. So far, only few GWAS have been performed on wheat for the resistance to soil-borne viruses, due to specific limiting factors. First, the exploitation of new genetic material is basic to find new resistance trait. The use of landraces and wild relatives of crop species can overcome this problem, since they contain wider genetic diversity than most cultivated genotypes [37]. Secondly, the evaluation of the damage caused by these viruses is challenging because their symptoms are similar to those produced by physiological stresses and are clearer after exposure of crops to low temperatures. The set up of new methods for the detection of soil-borne viruses in wheat plants is then an important aspect.

In the present work, we characterized the response to both SBCMV and WSSMV in 212 durum wheat genotypes, mostly landraces, to identify novel genetic material that could be interesting for finding new resistance traits. To this purpose, we set up a specific experiment in a screenhouse where the durum wheat plants were grown on *P. graminis* infested soil. The amount of both SBCMV and WSSMV RNAs in leaves was quantified through quantitative real time (qRT)-PCR (qPCR from now on) to classify the genotypes for their resistance/susceptibility to SBCMV and to WSSMV, separately. In addition, we performed a pilot GWAS to evaluate the use of qRT-PCR in association studies and to detect novel genetic regions that may be implicated in the resistance to these viruses.

2. Materials and methods

2.1. Plant material

We selected 211 genotypes (Table S1) from the Global Durum Wheat Panel germplasm collection (GDP) [38]. The GDP consists of a wide representation of *T. turgidum* ssp. *durum* modern germplasm and landraces. The genotypes selection has been performed to maintain the GDP genetic diversity: to maximize biodiversity, the chosen genotypes consisted in 189 landraces (out of the 192 landraces present in the GDP) and 22 modern varieties. In addition, the cultivar Claudio, that is not part of the GDP, was included in the screenhouse experiment, since it is often considered as reference for SBCMV infection studies [24,25]. A total of 212 genotypes were then considered in our study.

2.2. Population structure analysis

We considered the 211 genotypes included in the GDP for population structure analysis. Single nucleotide polymorphism (SNPs) data were downloaded from the GrainGenes database (https://wheat.pw.usda.gov/GG3/). Briefly, they were genotyped using the Illumina iSelect 90 K SNP array technology [39] and described by Mazzucotelli et al. [38]. The SNP data were filtered to remove markers with allele frequencies lower than 5 % (Table S1).

To establish the number of groups in our population, a discriminant analysis of principal components (DAPC) was performed using the 'adegenet' package 1.4.1 [40] in RStudio 4.2.2 (https://posit.co/products/open-source/rstudio/). The decreasing rate of the Bayesian information criterion (BIC) was visually inspected, then four clusters were selected taking the value of k above which BIC values decreased. After cluster number selection, an analysis of admixture by kinship Bayesian clustering technique was used to classify the group of genetically similar population via statistical software STRUCTURE v.2.3 [41]. Burn-in iterations of 10,000 cycles, followed by a simulation run of 100,000 cycles, admixture model, and 4 as k value were set with 11 independent runs.

2.3. LD Decay

Linkage disequilibrium (LD) was calculated using TASSEL5 and

measured using r^2. SNP dataset has been used to calculate pairwise marker correlation (r^2) for each chromosome, considering SNPs with an MAF >0.05, using TASSEL5 [42]. The r^2 of unlinked markers was estimated as the 95th quantile of r^2 values of markers on different chromosomes. LD decay curve was fitted using non-linear model [43]. To estimate the local LD value along chromosomes, each marker LD was calculated using the full matrix LD type for every combination of sites in the alignment. The LD decay was determined by plotting the r^2 values against the genetic distance of loci pairs (Mb) for each chromosome and a trend line describing the LD decay was calculated by locally weighted polynomial regression (LOESS) in R. The intersection point between the LD curve and the r^2 threshold determined the LD decay.

2.4. Phenotyping

2.4.1. Preparation and characterization of the inoculum

Soil infested with *P. graminis* carrying SBCMV and WSSMV was collected at an experimental station at the University of Bologna, Cadriano (Italy) in a field used in several previous studies for testing SBCMV resistance of wheat cultivars [17,24,35,44] and for developing virus fast detection methods [45,46]. The presence of *P. graminis* in the soil was confirmed by PCR according to [47] (Figure S1).

To determine which viruses can be transmitted to wheat through the soil, the virome of plants grown in the field was analysed. Young symptomatic and asymptomatic leaves were harvested, and total RNA was extracted (see below). Equal amounts of total RNA were pooled from 20 randomly selected samples (10 symptomatic and 10 asymptomatic) and subjected to 150 bp paired-end RNA-seq Illumina sequencing, after ribosomal depletion (Novogene, UK). Raw reads were processed with fastp v. 0.21.0 [48], using default parameters, for quality check and removal of adapters. Residual ribosomal sequences were discarded using bbduk script (default parameters) from bbmap v. 38.7 [49]. Reads were then assembled using metaspades v. 3.15.1 [50] and the obtained contigs were further extended with CAP3 (VersionDate: 02/10/15) [51]. Viral contigs were annotated using Diamond blastx (v. 2.0.15), against the non-redundant sequences database available at the NCBI (https://www.ncbi.nlm.nih.gov/).

Since a uniform concentration of the vector and the viruses cannot be guaranteed in different parts of the field, we prepared a homogeneous substrate for our experiments with the help of a cement mixer: 500 Kg of infested soil were thoroughly mixed with sterilized commercial soil and sterilized sand in a 1:1:1 ratio, thus obtaining a homogeneous substrate for the experiment. Approximately 3,6 L of this substrate was then placed in pots (15X15x20 cm).

2.4.2. Set up of the natural transmission experiment

A single experiment was conducted in a semi-controlled environment with the characterized infested soil. The seeds of the 189 landraces and the 23 cultivars of durum wheat were surface-sterilized and planted, in the previously prepared pots, in autumn (26–27 October 2021), consistently with the seasonal growth of durum wheat. The pots were then randomly positioned in a screenhouse protected only by an insect-proof net, inspected every week and watered whenever necessary to maintain high humidity in the soil, a condition optimal for the vector's activity. Pots were supplied with granular slow-release fertilizer YaraMila Blustar (Yara Italia S.p.A., Milano, Italy) at the beginning of the experiment. For each genotype, two pots were utilized, each containing six plants, *i.e.*, a total of 12 plants/genotype. Temperature was monitored with a data logger (Figure S2). From January, all the plants were weekly controlled to monitor the onset of virus-induced symptoms. Four months after the start of the experiment (1–3 March 2022), symptoms were visually scored. At that date, plants were at the stage 4 or 5 in the Feekes scale [52], depending on the genotype. Based on the average intensity of the symptoms observed in each pool of 12 plants, a disease severity index (DSI) was assigned to each genotype, similarly to the classification previously used by other authors [53]. The DSI was from 0 to 4 for crescent symptoms: 0 indicated no visible symptoms; 1 indicated very mild symptoms; 2 indicated mild mottling and stunting; 3 indicated mottling and stunting, yellowing; 4 indicated severe mottling and stunting with occasional plant death. This simplified categorization was necessary to mediate the variations among the 12 individuals of the same genotype and to reduce biases due to subjective judgement, which was in any case expressed by two persons.

After scoring, a young leaf was collected from each plant, corresponding to 12 leaves for each genotype. The 12 leaves were randomly grouped into three replicates, each consisting of four leaves. All samples were stored at −80 °C.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from durum wheat leaves using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich Pty. Ltd., Sydney, NSW, Australia) according to manufacturer instructions. RNA was eluted in 50 μL of nuclease-free water and 1 μg was used for cDNA synthesis with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Cheshire, UK) in a final volume of 20 μL, according to manufacturer instructions.

2.6. Viral load evaluation

Quantification of viral load was performed on the cDNAs by qPCR with specific primers for SBCMV (primer pair qPCR_577fw/qPCR_714rv) [45] and for WSSMV (primer pair WSSMV-L1/WSSMV-R1 [54].

The qPCR mix consisted of 1 μl of template cDNA, 0.15 μL of each primer, 3.7 μl of nuclease-free water and 5 μL of 2X iTaq Universal SYBR Green Supermix (Bio-Rad) in a final volume of 10 μl. Thermal profile of the reaction was an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 5 seconds and 60 °C for 30 seconds, concluding with 70 °C for 5 minutes. Melting curve was performed from 65 to 95 °C (increment 0.5 °C). The reaction was performed using the CFX96 Real-time PCR Detection System thermal cycler (Bio-Rad, Hercules, CA, USA) and controlled by the CFX Maestro software. In each plate, a negative control (NC) and a no template control (WC) were added, and amplification efficiency (E) was determined using Bio-Rad's Maestro software, according to the standard curve generated from a dilution series (six dilution points) of a positive control. All reactions were performed with three technical replicates.

The Pfaffl method was used to calculate the relative viral load [55]. The elongation factor (EF1 α) was used as reference gene [56], while the genotypes GDPv2_717 and GDPv2_577 were used as calibrator for SBCMV and WSSMV, respectively [55]. When viral RNA was not detectable, *i.e.*, in case of expression level below the detection threshold, an arbitrary Ct value of 35 was used for calculation.

The viral load of each virus measured by qPCR was expressed as $\log_2$ Viral Expression Ratio (VER). For each virus, we defined six classes of viral load based on $\log_2$VER values: i) class 1, for very low viral load ($\log_2$VER<0); ii) class 2, for low viral load ($0 <\log_2$VER<5); iii) class 3, for medium viral load ($5 <\log_2$VER<10); iv) class 4, for medium/high viral load ($10 <\log_2$VER<15); v) class 5, for high viral load ($15 <\log_2$VER<20); vi) class 6, for very high viral load ($\log_2$VER>20).

R scripts were used for hierarchical clustering of genotypes according to viral load and to calculate Kendall correlation (Supplementary File 1). The non-parametric Kruskal-Wallis test was used to evaluate statistically differences in symptomatology among clusters.

2.7. Genome wide association study

Since the relative viral load obtained using the Pfaffl method resulted very left skewed for both SBCMV and WSSMV (Figure S3), the best-Normalize R package [57] was used to find the best transformation to normalize these qPCR data effectively. The best normalization was

obtained with the technique called Ordered Quantile normalization (orderNorm, or ORQ). The normality of the data was assessed using the Shapiro-Wilk normality test (data not shown). Normalized data were used in both TASSEL5 software [42] and mrMLM R package [58]. This package includes six multi-locus GWAS models: mrMLM (multi- locus random-SNP-effect MLM) [59], FASTmrMLM [60], FASTmrEMMA (fast multi-locus random-SNP-effect efficient mixed model analysis) [61], ISISEMBLASSO (iterative modified-sure independence screening expectation-maximization-Bayesian least absolute shrinkage and selection operator) [60], pLARmEB (polygenic-background-control-based least angle regression plus empirical Bayes) [62] and pKWmEB (integration of Kruskal-Wallis test with empirical Bayes) [63].

For TASSEL5, the threshold level for significant marker-trait associations was -$\log_{10}$(p-value) $>=$ 3. For the six multi-locus models, a critical threshold for significantly associated SNPs was set at LOD $>=$ 3.0. Statistically significant SNPs detected by at least two different algorithms of mrMLM and in TASSEL were considered as Quantitative Trait Nucleotides (QTNs). According to LD decay block previously calculated, genomic regions surrounding the QTNs were identified, and the list of candidate genes was compiled using Ensembl Biomart (release 56 - Feb 2023). The Svevo genome (version 1) available on Ensembl-Plants (https://ftp.ebi.ac.uk/ensemblgenomes/pub/release-56/plants/gff3/triticum_turgidum) was used for this purpose [64].

GO enrichment was performed using g:Profiler webtool (https://biit.cs.ut.ee/gprofiler/gost).

3. Results

3.1. Summary statistics of SNP markers, LD Decay and Population structure analyses

Among the 16,633 SNPs used for genotyping of the GDP [38], markers with minor allele frequency < 0.05 and monomorphic markers were eliminated, resulting in 13,551 high quality informative SNPs which were used for further analysis. In the selected population, the critical r^2 value corresponded to 3.8 Mbp (Figure S4). This data indicated that the genetic diversity of the landraces of the original population was maintained in the panel selected form the GDP for this study.

STRUCTURE analysis was employed to obtain the information on the population structure of the 211 durum wheat genotypes. Four clusters were identified, based on BIC decreasing criterion; a K= 4 was chosen taking the value of k above which BIC values decreased (Figure S5).

3.2. Detection of the soil-borne viruses for the infection experiment

Before performing the experiment in semi-controlled conditions, we evaluated if the selected soil could ensure a correct infection by soil-borne viruses of durum wheat plants. First, we confirmed the presence of the vector *P. graminis* in the soil collected at the experimental station at the University of Bologna, Cadriano (Italy) [17,24,35,44–46] (Figure S1). Secondly, we evaluated which viruses can directly infect plants grown in the experimental field. We performed an Illumina RNA sequencing and bioinformatic analyses of pooled symptomatic and asymptomatic leaves directly collected from durum wheat plants grown in the experimental field. The complete genomic sequences of SBCMV and WSSMV were reconstructed and deposited in the GenBank with Acc. Nos. OQ427639 (SBCMV RNA1), OQ427640 (SBCMV RNA2), OQ945144 (WSSMV RNA1) and OQ945145 (WSSMV RNA2). No other viruses were detected, so we concluded that the virome present in the wheat plants of the here described experiment was composed only by SBCMV and WSSMV.

3.3. Evaluation of SBCMV and WSSMW infection in the durum wheat genotypes

The 212 genotypes were grown in a screenhouse in semi-controlled conditions, as described in Material and Methods, all having the same inoculum and therefore the same opportunity to be colonized by *P. graminis*, and under seasonal conditions that mimic real field conditions. The experiment in semi-controlled conditions was initiated at the end of October 2021, consistently with the seasonal growth of durum wheat. Plants were visually inspected every week. The first viral symptoms appeared in February and persisted until May (Fig. 1). As reported by several authors, the two viruses induce similar phenotypes with mottling and yellowing on leaves and reduced size of plants, therefore it was not possible to distinguish between the symptoms induced by SBCMV and those induced by WSSMV [3,19]. Symptoms were visually scored on 1–3 March 2022, *i.e.*, four months after the start of the experiment. At that date plants were at the stage 4 or 5 in the Feekes scale [52], depending on the genotype. Based on the intensity of the symptoms, a disease severity score from 0 to 4 was assigned to each genotype according to the index described in Materials and Methods (paragraph 2.4.2): i) no symptoms (DSI=0) were detected in 65 genotypes (30.7 % of the 212 genotypes); ii) very mild symptoms (DSI=1) were detected in 83 genotypes (39.2 %); iii) mild mottling and stunting (DSI=2) were observed in 36 genotypes (17 %); iv) mottling and stunting (DSI=3) were observed in 23 genotypes (10.9 %); v) severe mottling and stunting with occasional plant death (DSI=4) were observed in five genotypes (2.4 %; Fig. 2; Table S2).

After symptoms evaluation, young leaves from each plant were sampled, total RNA was extracted, and cDNA was synthetized. The viral load of each virus was measured by qPCR and for each virus, six classes of infection were defined based on $\log_2$VER values, as described in Materials and Methods (paragraph 2.6; Fig. 3).

The viral load of SBCMV exhibited a high differentiation among the genotypes (Fig. 3, Table S2, Figure S6): 28 genotypes, including Meridiano, Cappelli and Svevo, which are known to be resistant to this virus [44], belonged to class 1; 56 genotypes, including Claudio, which is known to be moderately susceptible to this virus [24], belonged to class 2; 62 genotypes belonged to class 3; 20 genotypes belonged to class 4; 11 genotypes, including Simeto, which is known to be susceptible to this virus [44], exhibited a high viral load and belonged to class 5; and 35 genotypes belonged to class 6, showing a very high viral load.

Quite different was the case of WSSMV, where no genotypes showed viral load values under the PCR detection limit or a $\log_2$VER value below 0, but most genotypes had a very high viral load (Table S2, Fig. 3 and S7). In detail, 33 genotypes belonged to class 2, with a low viral load; 13 genotypes belonged to class 3, with a medium viral load; 10 genotypes belonged to class 4, showing a medium/high viral load; 89 genotypes, including Meridiano, Svevo, Claudio and Simeto, belonged to class 5, showing a high viral load; and 67 genotypes, including Cappelli, showed a very high viral load and belonged to class 6 (Fig. 3 and S7).

We evaluated the possible presence of a correlation between the viral load of each virus and the above reported DSI. For this purpose, we chose the Kendall correlation test since it is a non-parametric test useful for quantitative paired measures and suitable for data that don't have a normal distribution, as the qPCR data here reported. The Kendall correlation was τ= 0.13 for SBCMV, and slightly higher (τ=0.30) for WSSMV, indicating a very low correlation between each infection and the visualized symptoms.

Considering both viruses, our data showed that many of the genotypes accumulated very low to medium amounts of SBCMV but high to extremely high amounts of WSSMV (Fig. 3). We then investigated if the infection with the two viruses could have a synergistic effect in term of viral load. The low Kendall correlation value obtained (τ=0.22) considering the $\log_2$VER values did not indicate a clear correlation between the viral loads of the two viruses.

Hierarchical clustering of the genotypes, according to their viral load, led to the identification of five clusters (cl1, cl2, cl3, cl4, cl5; Fig. 4 and Supplementary Table S2). The cl1, cl4, cl2 were associated to high WSSMV viral load and different levels of SBCMV viral load. Cl1 grouped most of the genotypes (113), including Cappelli, Svevo, Meridiano, and

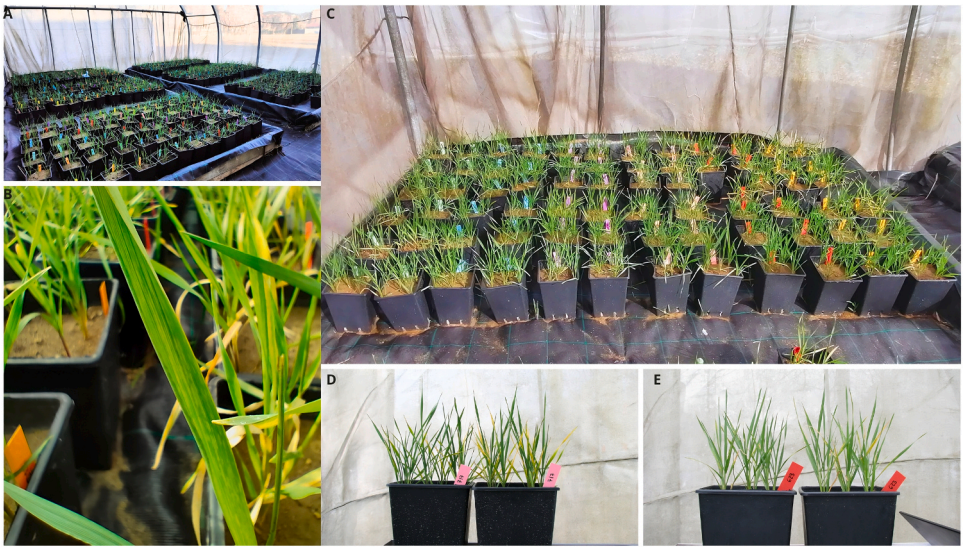

Fig. 1. Natural transmission experiment of SBCMV and WSSMV in screenhouse semi-controlled conditions. A) Plants after three months from sowing. B) Typical symptoms associated with SBCMV and WSSMV infection after four months from sowing. C) Overview of plants at the time of sampling, four months after sowing. D) Plants of the genotype GDP_717 at the time of sampling. E) Plants of the genotype GDP_623 at the time of sampling.

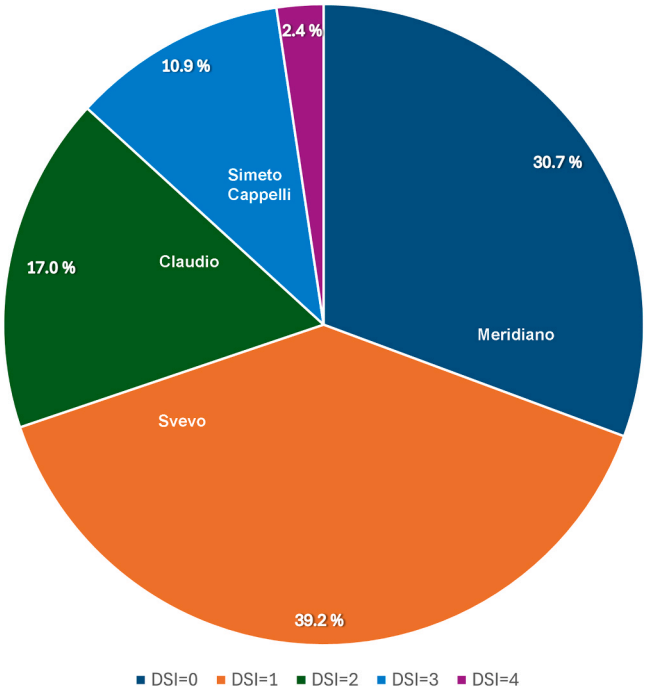

Fig. 2. Distribution of symptomatology according to disease severity index (DSI). 0 = no visible symptoms; 1 = very mild symptoms; 2 = mild mottling and stunting, 3 = mottling and stunting, yellowing; 4 = severe mottling and stunting with occasional plant death.

Claudio, that were characterised by high WSSMV and low SBCMV viral loads. Cl4 consisted in 29 genotypes with high WSSMV and medium SBCMV viral loads, whereas cl2 consisted in 24 genotypes, including Simeto, with both high WSSMV and SBCMV viral loads. Differently, cl3 and cl5 showed low levels of WSSMV viral load: cl3 grouped 42 genotypes that were characterised by low viral load for both viruses, whereas cl5 was constituted by only four genotypes with high SBCMV viral load and low WSSMV viral load (Fig. 4 and Table S2).

Interestingly, 23 genotypes exhibited very low or low levels of both viral loads (corresponding to $\log_2$VER class 1 or 2, respectively) and absent or reduced symptoms (corresponding to DSI 0 or 1, respectively) and could be considered resistant to both SBCMV and WSSMV (Table 1). In particular, SBCMV viral load was not detectable in the seven genotypes FIGSDWHOTCLD166, SSD 255, SSD 244, Vendetta, MARVARID, ISTIQBOLLI, FIGSDWHOTCLD149 (highlighted in bold in Table 1; Table S2).

3.4. *Association analyses for the response to SBCMV and WSSMV*

To identify possible genetic determinants that may be involved in the infection of the two soil-borne viruses in durum wheat plants, we used the normalized qPCR data, *i.e.*, the $\log_2$VER values, for both single-locus and multi-locus GWAS. The latter method is known to be characterized by high power and accuracy, and the comparison of the two methods allow to confirm the results obtained from each analysis.

Regarding SBCMV infection, the single-locus GWAS identified six significant associated SNPs (Table S3) and the multi-locus GWAS identified eight significant SNPs, of which two were identified by at least two different models (Table S4). The same pipeline was used for WSSMV infection data. The single-locus GWAS identified 20 significant SNPs (Table S5), whereas the multi-locus GWAS identified 11 SNPs associated to WSSMV infection, of which four were identified by at least two different models (Table S6).

We then compared the results obtained with single-locus and multi-locus GWAS and considered for further evaluation those QTNs that resulted significant in both analyses, *i.e.*, one for SBCMV analysis and two for WSSMV analysis, as reported in Table 2.

The average of trait variance between Tassel and mrMLM was 6.7 % for the marker Kukri_rep_c107605_569, related to SBCMV, while 8.2 % and 5.5 % for the markers BS00076872_51 and BS00065082_51, respectively, related to WSSMV (Table 2).

The chromosomal distribution of the three retained QTNs is shown in Fig. 5. According to the calculated LD decay, the surrounding chromosomal regions were considered for each of the three retained QTNs and the genes included in the considered regions were identified (Table S7). For the QTN associated to SBCMV resistance (Kukri_rep_c107605_569), 57 genes were found in the confidence interval (Table S7A). For the QTNs associated to WSSMV resistance (BS00076872_51, and BS00065082_51), 75 and 46 genes were found in the confidence intervals, respectively (Table S7B and Table S7C).

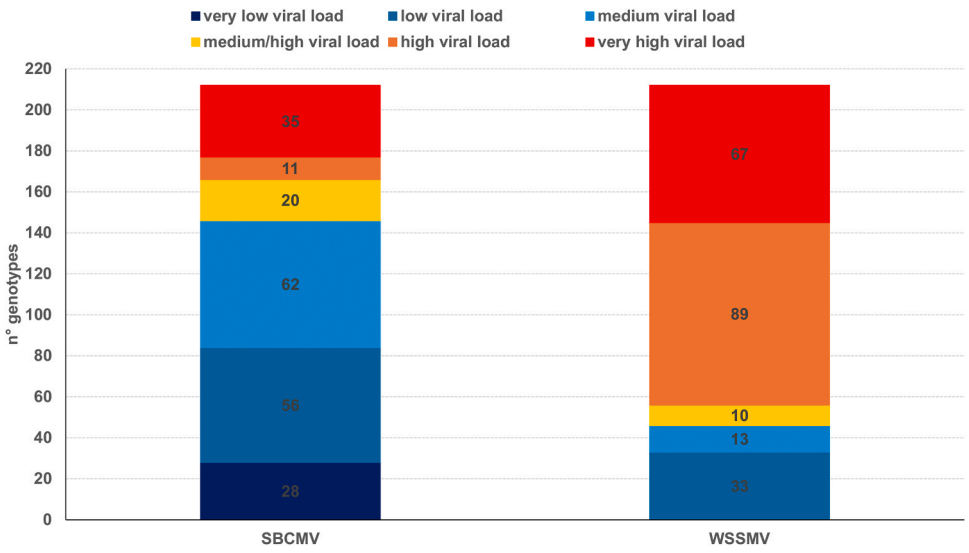

Fig. 3. Virus accumulation in the 212 genotypes. Distribution of the classes related to the SBCMV and WSSMV viral loads: dark blue: very low viral load (log_2VER<0); blue: low viral load (0 <log_2VER<5); light blue: medium viral load (5 <log_2VER<10); yellow: medium/high viral load (10 <log_2VER<15); orange: high viral load (15 <log_2VER<20); red: very high viral load (log_2VER>20). For each virus, the number of the genotypes belonging to each class is shown.

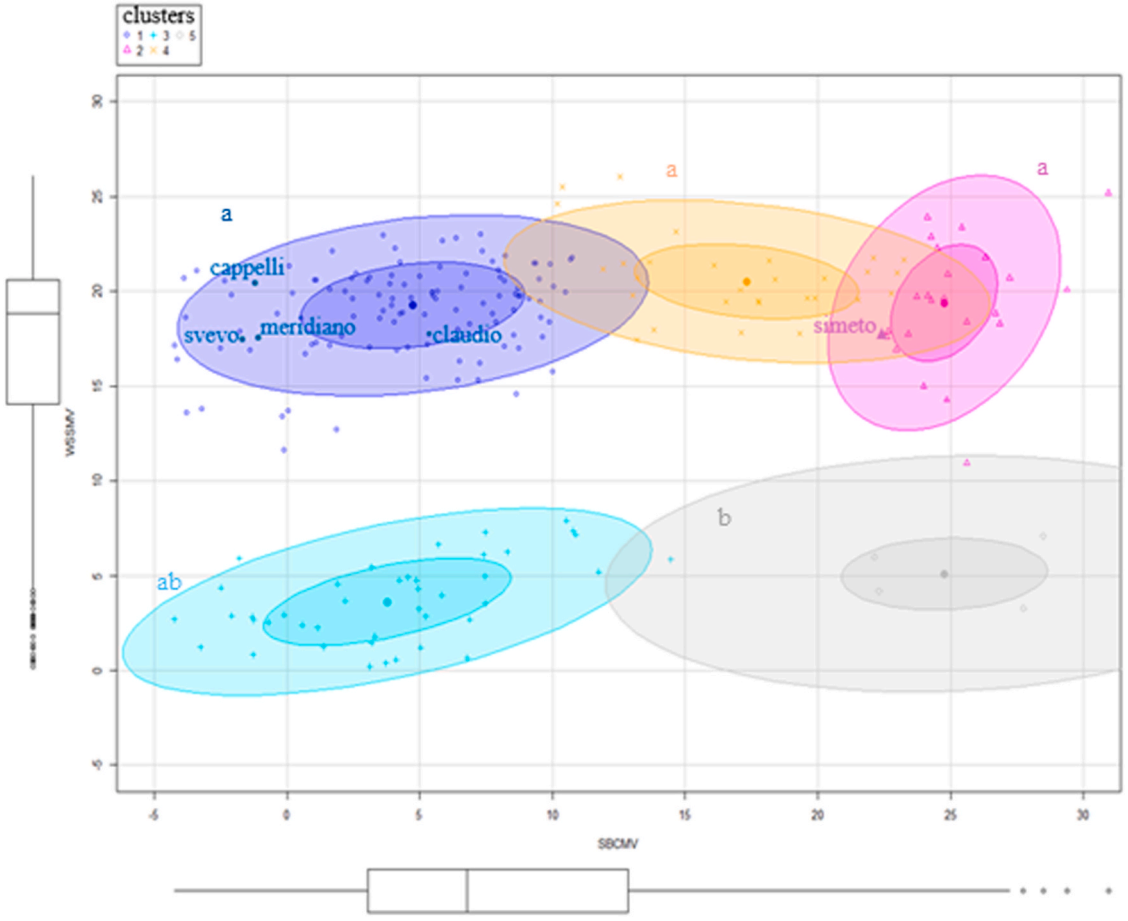

Fig. 4. Scatter plot of SBCMV and WSSMV viral loads. Clusters are highlighted by different colours. Central points represent the average value for each cluster, the internal and external ellipses represent confidence intervals of 0.95 % and 0.5 %, respectively. Marginal box plots represent the distribution of SBCMV and WSSMV, respectively; lines in the box correspond to the median, margins of the box correspond to the first and third quartiles. Different letters indicate statistically significant differences according to the Kruskal-Wallis non-parametric test (p-value 0.05).

Table 1
List of genotypes selected as resistant to SBCMV and WSSMV (log_2VER<5 and DSI 0 or 1). Accessions where SBCMV viral load was not detectable are in bold. VER: Viral expression ratio.

Accession Code	Accession Name	SBCMV log_2 VER	Class of SBCMV log_2 VER	WSSMV log_2 VER	Class of WSSMV log_2 VER	Disease Severity Index (DSI)
GDPv2_629	**FIGSDWHOTCLD166**	−4.249175701	1	2.701287492	2	0
GDPv2_686	**SSD 255**	−3.260155874	1	1.218559391	2	0
GDPv2_683	**SSD 244**	−2.494191774	1	4.358143433	2	0
GDPv2_379	**Vendetta**	−2.093082062	1	2.871427966	2	1
GDPv2_739	**MARVARID**	−1.308448439	1	2.825302991	2	0
GDPv2_742	**ISTIQBOLLI**	−1.277367538	1	0.815668679	2	0
GDPv2_628	**FIGSDWHOTCLD149**	−0.695957259	1	2.521436606	2	0
GDPv2_661	SSD 107	−0.108712395	1	2.918230903	2	0
GDPv2_703	SSD 400	0.577844063	2	2.37200189	2	0
GDPv2_757	Hordeiforme 27	1.156228765	2	2.255191535	2	1
GDPv2_621	FIGSDWHOTCLD090	1.377876489	2	1.230342322	2	0
GDPv2_714	SSD 470	1.893031985	2	4.533329669	2	0
GDPv2_659	SSD 069	2.17979127	2	3.66490112	2	1
GDPv2_577	Karabalykskaya chernokolosaya 20	3.10629343	2	0.195103324	2	1
GDPv2_692	SSD 278	3.189975017	2	1.443652996	2	0
GDPv2_688	SSD 262	3.30025962	2	1.78298002	2	0
GDPv2_608	FIGSDRYWET115	3.717868351	2	0.368231057	2	0
GDPv2_678	SSD 188	4.110621775	2	0.551052338	2	0
GDPv2_644	SSD 011	4.232458225	2	4.741460868	2	1
GDPv2_755	Azulejo	4.543714041	2	4.901792159	2	0
GDPv2_576	ordeiforme 254	4.862991687	2	4.739420294	2	1
GDPv2_761	IQ 118	4.926386905	2	4.303561286	2	0
GDPv2_733	RUSCIA	4.97166851	2	3.24083715	2	1

Table 2
List of the SNPs identified by both TASSEL and mrMLM (at least two models) analyses. Chr: chromosome. Position: physical position in base pairs according to the Svevo v1 reference genome. -log_{10}(p): negative logarithm in base 10 of the p-value. For QTN effect, the positive or negative value indicates an increasing or decreasing effect of the minor allele on the trait. r^2(%): percentage of phenotypic variance explained by the marker.

Trait	SNP name	Chr	Position (bp)	Tool	Algorithm	-log_{10}(p)	QTN effect	r^2 (%)
SBCMV	Kukri_rep_c107605_569	4B	610,745,046	TASSEL	MLM	3.8	/	6.37
				mrMLM	mrMLM	5.43	0.30	8.62
					FASTmrMLM	5.06	0.24	5.52
					pLARmEB	6.32	0.23	5.14
					pKWmEB	3.79	0.15	7.77
WSSMV	BS00076872_51	6 A	572,223,986	TASSEL	MLM	4.21	/	8.40
				mrMLM	mrMLM	4.65	−0.37	7.56
					FASTmrMLM	5.87	−0.36	7.64
					pKWmEB	5.54	−0.30	9.04
	BS00065082_51	4B	553,851,296	TASSEL	MLM	3.18	/	6.14
				mrMLM	mrMLM	5.16	−0.50	6.99
					pLARmEB	4.10	−0.36	3.85
					pKWmEB	4.25	−0.33	6.29
					ISIS EM-BLASSO	5.04	−0.39	4.47

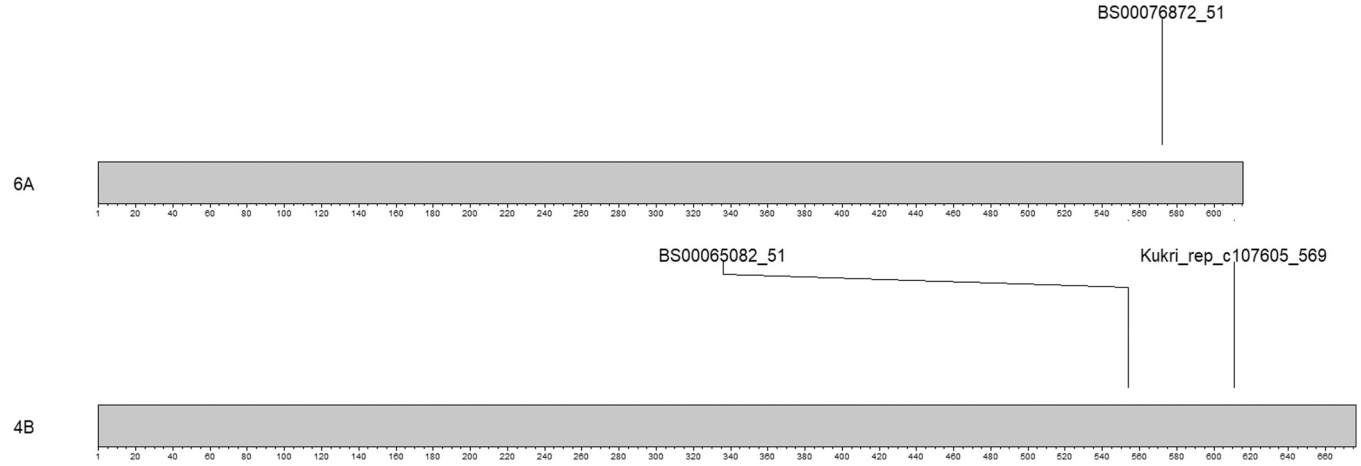

Fig. 5. Positions of the three significant QTNs on the durum wheat chromosomes 6 A and 4B. The distances in the chromosomes are expressed in Mbp.

4. Discussion

4.1. Natural transmission of the soil-borne viruses SBCMV and WSSMV in durum wheat plants under semi-controlled conditions

In this study a panel of 212 durum wheat genotypes was used to perform a single experiment of natural transmission of the soil-borne viruses SBCMV and WSSMV under semi-controlled conditions in a screenhouse, to evaluate their susceptibility and find putative genetic determinants involved in the resistance to these two soil-borne viruses. We selected 211 genotypes from the GDP [38] and included mostly durum wheat landraces to maximize biodiversity. Indeed, it is known that landraces and wild relatives serve as a major reservoir for different traits, including virus resistance [32,37].

A key condition for the study of the interaction between plants and soil-borne viruses is to guarantee optimal conditions for the activity of the vector. In our experiment this result was enabled by the employed experimental setup, which included the use of uniformly mixed infested soil and periodic irrigation. This experimental system allowed us to circumvent several problems that are common in field experiments, including the non-uniform distribution of both vector and viruses, possible differences in soil composition in different parts of the field, and the lack of sufficient rainfall during winter periods, that is a limiting factor for *P. graminis* activity. These factors frequently compromise the outcomes of field experiments, requiring field trials over several years [32]. Here, the successful transmission of WSSMV in all wheat genotypes (Fig. 3 and Table S2) demonstrated that the conditions for soil-borne viruses' transmission were favourable and supports the reliability also of the results obtained for SBCMV.

Up to now, a limited number of studies used soil from infested fields to screen wheat germplasms for resistance to the closely related species SBCMV and SBWMV [20,26]. In these studies, genotypes of *Triticum aestivum* and *T. monococcum* were employed, and the tests were conducted under controlled conditions in growth chambers with a fixed 16 h photoperiod and temperatures between 16 and 20°C [20] or between 16 and 18°C [26] with no natural oscillations of the temperature or day length. Differently, our experiment in the screenhouse relied on the external temperatures of Northern Italy frequently reaching temperatures below zero in December and January during the night, and with ample daily thermal excursions, particularly evident in February (Figure S2). Then, our approach allowed to have the natural field conditions for infection, in addition to inoculum homogeneity. Consistently, the analysis of the soil showed the presence of *P. graminis* and the analysis of the plant tissues grown in this soil confirmed that solely SBCMV and WSSMV were present.

4.2. Evaluation of qPCR for the determination of viral load in wheat samples

The evaluation of symptoms is a key aspect in the study of virus resistance in crops. Usually, the level of infection is evaluated through visual scoring [32]. Unfortunately, in the case of soil-borne viruses, symptomatology can be misleading because it is not possible to discriminate which virus is the causal effect of the symptoms. Moreover, the symptoms caused by these viruses are similar to those produced by abiotic or nutritional stresses.

A common technique for plant virus diagnosis and detection is the ELISA [24,44], frequently used in the last decades for its specificity, simplicity and affordability, even if it is more suitable for qualitative than quantitative evaluation [65]. In recent years, molecular approaches with a higher sensitivity, such as qPCR, became available [65–67]. A real-time qPCR protocol for SBCMV in winter wheat was already described: when compared to the DAS-ELISA protocol, it proved 1000 times more sensitive [68]. Similarly, qPCR has been already used to screen rice cultivars for virus resistance [69]. We then decided to use qPCR to screen our wheat genotypes, considering that it is more sensitive than ELISA and the data obtained have an extended range that can be used for GWAS. A comparison between qPCR and ELISA data would not be useful, since a positive result by qPCR could not be confirmed by ELISA, due to the lower sensitivity of this method. This analysis allowed us to quantify the viral RNA present in the leaf samples and classify the analyzed genotypes based on the viral load of the two viruses separately. To confirm our data, we compared the qPCR results of SBCMV infection related to five analyzed genotypes, *i.e.*, Meridiano, Cappelli, Svevo, Claudio, Simeto, with the data present in literature, since these five cultivars are the only ones present in our panel that had been extensively analyzed in previous studies for SBCMV infection [24,25,44,70] (Table S8). Consistently, in our experiment Simeto resulted susceptible, Claudio moderately susceptible and Meridiano, Cappelli and Svevo resistant, as previously reported [24,25,44]. A recent study on SBCMV resistance considered Meridiano and Svevo as the reference resistant genotypes and Claudio as the reference susceptible genotype [35]. In conclusion, our study confirmed the reliability of qPCR data to quantitatively measure the infection of SBCMV and WSSMV in durum wheat.

4.3. In vivo evaluation of soil-borne viruses' infection on the durum wheat genotypes

The qPCR analysis highlighted the different behaviour of SBCMV and WSSMV in relation to the viral accumulation in plant leaves. SBCMV viral load was highly differentiated among the genotypes, with undetectable viral load in some genotypes, and viral load levels that spanned from very low to very high in others (Fig. 3). Differently, WSSMV was present in all the 212 genotypes, and most of them exhibited high or very high viral load (Fig. 3).

To our knowledge, only one previous study reported a field test where both viruses were analysed [14]. The authors compared the results of that study conducted with both viruses with those obtained on the same varieties in another environment where only SBCMV was present and concluded that WSSMV did not induce a loss of resistance to SBCMV [14]. In our assay, WSSMV was detected in all genotypes; the cultivars known to be SBCMV-resistant maintained this phenotype even in co-infection with WSSMV, confirming that WSSMV does not cause a breakdown of SBCMV resistance. Consistently, we searched for a possible correlation between the two viral loads, but no clear correlation could be detected, as shown by the low Kendall correlation value obtained. We also searched for possible correlations between each viral load measured in leaf samples through qPCR and symptoms evaluation. Very low correlation values were calculated for both viral loads. The presence of two viruses in the soil did not allow us to evaluate whether the symptoms were due to SBCMV or WSSMV or both. These data highlighted the limits in defining the infection level of plants only based on the evaluation of symptoms and strengthen the importance of using virus-specific detection methods for infection evaluation, such as qPCR analysis.

Plotting SBCMV *vs* WSSMV viral load for all genotypes evidenced clustering of genotypes (Fig. 4 and Table S2). Interestingly, cl3 grouped the genotypes that accumulated low levels of both SBCMV and WSSMV showing limited symptoms. In particular, our analysis identified 23 genotypes with low or very low viral loads of both SBCMV and WSSMV and absent or reduced symptoms (Table 1). These genotypes represent useful material for the study of the interaction between the plant and the two soil-borne viruses and to identify new resistance traits. In fact, breeding efforts for soil-borne virus resistance have so far focused mainly on SBCMV, while WSSMV did not receive much attention, even if it is present in North America and Europe and can cause a serious disease particularly in durum wheat [3,31].

4.4. GWAS for the response to soil-borne viruses

Finding genetic determinants for resistance is an important aim for

researchers and breeders, since the use of resistant cultivars is the most promising practice to avoid or limit crop yield loss. The challenge for plant breeders is to detect resistance in donors in a species diversity panel and then to use them to introduce the genes conferring resistance into elite but susceptible backgrounds. The forms of virus resistance can be monogenic or polygenic, and different authors underlined that SBCMV and WSSMV resistance are under polygenic or oligogenic control [24,31,36]. GWAS can be a powerful tool to accelerate mapping of both monogenic and polygenic resistance. So far, this kind of studies have rarely been used in the plant–virus interaction context [32]. This is likely due to the fact that GWAS requires continuous data while phenotyping data for virus resistance is often not continuous.

For resistance to SBCMV or SBWMV, few GWAS were performed so far in bread wheat [33,34] and durum wheat [35]. For WSSMV resistance, to our knowledge, only one GWAS was performed in bread wheat [36] and none in durum wheat. For this reason, the exploitation of GWAS on new genetic material may be very useful to identify novel genetic regions involved in resistance to these soil-borne viruses. The use of viral load-related qPCR data for the association analysis allowed to identify specific QTNs associated to SBCMV and WSSMV resistance.

4.4.1. Chromosomal regions associated to response to SBCMV

We found one QTN significantly associated to SBCMV response on chromosome 4B (Table 2). Some previously reported studies detected specific loci involved in SBCMV/SBWMV resistance. Three major loci have been mapped in bread wheat so far: *Sbm1* in the distal end of chromosome 5DL, which has been shown effective against SBWMV in the USA [71] and SBCMV in Europe [21]; *Sbm2* (short arm of chr. 2B), against SBCMV in Europe [23]; and *Sbm3* that was identified on the A genome of *T. monococcum* and successfully transferred to hexaploid wheat [26]. In durum wheat, Maccaferri and colleagues identified and fine mapped a major QTL for SBCMV resistance, the locus *QSbm.ubo-2B*, which coincides with *Sbm2*, and also identified other minor QTLs [24,25,35]. Our analysis did not detect any associated QTNs in the locus *QSbm.ubo-2B*. The detection of different QTNs for the same trait in diverse populations can be explained by several genetic, environmental, and methodological factors. In our case, the main explanation is the different population used in this study compared to previous ones. In particular, in the previous studies the authors used two panels, for a total of 549 genotypes, composed exclusively by varieties/breeding lines [24,35]. In contrast, in our study about 90 % of the genotypes are landraces. Hence, the different genetic background between our panel and those previously analysed may explain different genetic diversity, leading to variation in allele frequencies. Despite the lack in finding a QTN in the *QSbm.ubo-2B* locus, the QTN on chromosome 4B here identified as associated to SBCMV response is located in a region close to the confidence interval of a minor QTL identified by Maccaferri et al. [24]. This observation can be an indication that part of the genetic background could be shared between our genetic panel and those previously reported. This result also strengthens the reliability of the method used for phenotyping in our study, as it allowed us to identify a genomic region that was previously reported to be associated to SBCMV response with a different methodology (*i.e.*, ELISA).

4.4.2. Chromosomal regions associated to response to WSSMV

We found two QTNs significantly associated to WSSMV response on chromosomes 6 A and 4B (Table 2). In literature, one major QTL on chromosome 2DL and two minor QTLs on regions 5B and 7D were described for WSSMV resistance in bread wheat [27,36], whereas two main QTLs on chromosomes 7 A (*Qssm-mtpsa-7A*) and 7B (*Qssm-mtpsa-7BS*) and one minor QTL on chromosome 2 A were found in durum wheat [31]. In our analysis, no QTNs located in these chromosomes were found as significantly associated to both single-locus and multi-locus approaches. Interestingly, single-locus analysis detected three significantly associated SNPs on chromosome 7B (wsnp_Ex_c7783_13260929, BS00088495_51, wsnp_RFL_Contig3854_4205716; Table S5) in a physical zone not distant to one of the two confidence intervals of *Qssm-mtpsa-7BS* [31]. This observation confirmed that chromosome 7B can be important for WSSMV resistance.

Our results suggest that, beyond the already identified QTLs, other genomic regions could contain genetic determinants for the resistance to these two soil-borne viruses. Although our GWAS analyses are preliminary and need to be validated through further experiments, this work represents an important step forward, because it sets the basis for the identification of new genomic regions involved in the resistance to SBCMV and WSSMV, as well as associated candidate genes with a biological role in response to viral infection.

4.5. Searching for putative candidate genes involved in virus resistance

The identification of virus resistance genes is challenging because the interaction between plant and virus is very specific and highly dependent on both virus and plant species. Among the candidate genes found through GWAS, we searched those that may be involved in plant-virus interaction mechanisms, including those genes that are known to be involved in the signalling pathways [72,73].

Kinases are important effectors in the signalling pathway in response to pathogens, including viruses [73]. Consistently, we found six kinase-encoding genes (TRITD4Bv1G179940, TRITD4Bv1G180140, TRITD4Bv1G180320, TRITD4Bv1G180440, TRITD4Bv1G182010, and TRITD4Bv1G182520) on chromosome 4B that are putatively associated to SBCMV resistance, and four kinase-encoding genes putatively associated to WSSMV resistance, three on chromosome 6 A (TRITD6Av1G204270, TRITD6Av1G206140, and TRITD6Av1G206540) and one on the chromosome 4B (TRITD4Bv1G163300).

Many plant TF families, including NAC, MYB, zinc finger, WRKY, AP2/ERF, bZIP, and bHLH, are involved in the transcriptional activity of virus-responsive genes [72]. These TFs can also regulate Pathogen-Related (PR) genes. Consistently, we found several TF encoding genes in the considered QTNs. Regarding the SBCMV-related association, on chromosome 4B (Kukri_rep_c107605_569-related QTN) we found four zinc finger proteins (TRITD4Bv1G180550, TRITD4Bv1G181440, TRITD4Bv1G182330, TRITD4Bv1G182390), two putative LOB domain-containing proteins (TRITD4Bv1G180790, TRITD4Bv1G180800) and one ethylene-responsive TFs (ERFs, TRITD4Bv1G181190). Regarding the WSSMV-related associations, on chromosome 6A (BS00076872_51-related QTN) we found one TF GTE9-G (TRITD6Av1G205230), three ERF (TRITD6Av1G205400, TRITD6Av1G205410, TRITD6Av1G205420) and one MYB TF (TRITD6Av1G206230), whereas on chromosome 4B (BS00065082_51-related QTN) we found one zinc finger protein (TRITD4Bv1G161430), one winged-helix DNA-binding TF (TRITD4Bv1G161870) and one putative transcription repressor OFP12 (TRITD4Bv1G163410).

Although TFs are known to be involved in the response to both abiotic and biotic stresses, including virus infection, the regulatory role of TFs during viral infection is less understood compared to those against other pathogens [72]. Few studies are reported about the activity of TFs and virus response.

In the well-studied N resistance to tobacco mosaic virus (TMV) it was demonstrated that downregulation of NtMYB1 compromised the resistance [74]. In addition, silencing of the MYB SlMYB28 in tomato improved plant resistance to tomato yellow leaf curl virus (TYLCV) infection, suggesting that it acts as a negative regulator for viral infection control [75]. Moreover, some ERF TFs have been previously described for their involvement in the response to viral infection. Interestingly, the overexpression of tobacco gene NtERF5 enhanced resistance to TMV [76]. Another ERF TF, Tsi1, when overexpressed in transgenic hot pepper plants enhanced host resistance to different pathogens, including viruses, through the overexpression of PR genes [77].

Positive-strand RNA viruses carry a small genome that encodes a limited set of functional proteins, insufficient to complete the entire

virus replication and proliferation cycle. For this reason, during evolution viruses developed the ability to recruit host factors to complete their infectious life cycle. This dependence on host factors gives to plants the possibility to evolve mechanisms to limit or counteract the consequences of viral infection. On the other hand, the absence of essential host factors could interfere with the infection process or restrict viral proliferation leading to either mono- or polygenic recessive resistance [78]. This bipartite relationship is very specific and differs from one virus to another, thus making it very difficult to identify the susceptibility or resistance factors involved. For this reason, among the genes belonging to the considered chromosomic regions, we searched for those possibly involved in virus replication and proliferation, since they can represent interesting candidates for virus resistance.

4.5.1. Candidate genes for SBCMV resistance

In the region on chromosome 4B associated to SBCMV response, different genes could be involved in in virus replication and proliferation (Table 3).

The RNA-binding protein TRITD4Bv1G179760 could contribute to host immunity against RNA virus infection, through either host RNA regulation or viral RNA degradation. Many examples are reported about RNA-binding proteins that function in sequence-specific or non-specific manner [79].

The gene TRITD4Bv1G180460 codes for a defensin. These proteins are effectors of the innate immune system with potent antibacterial, antiviral, and antifungal activity. In animals, defensins have direct antiviral activity in cell culture, with varied mechanisms for individual viruses, although some common themes have emerged. In addition, defensins have potent immunomodulatory activity that can alter innate and adaptive immune responses to viral infection [80,81]. Whether defensins are involved in antiviral resistance in plants remains to be determined [82].

The gene TRITD4Bv1G180870 codes for a Hypersensitive Response (HR)-like lesion-inducing protein that is putatively involved in hypersensitive response, as reported in *Nicotiana benthamiana* for a (HR)-Like Lesion Inducing Protein 4 named NbHRLI4 in response to turnip mosaic virus [83].

TRITD4Bv1G181320 codes for a Appr-1-p processing enzyme family protein. In humans, an appr-1-p processing enzyme protein has been shown to be involved in viral RNA replication [84]. The host/substrate-specific activity of Appr-1″-pase might provide a selective advantage in viral replication or/and modulation of host-factors during natural infection. TRITD4Bv1G181320 may act in durum wheat against virus infection in a similar manner, even if the X-domain's Appr-1″-pase activity or homologs are identified in animal RNA viruses but not in plant viruses, so far.

Specific DET1-and DDB1-associated proteins, like TRITD4Bv1G182080, can be involved in direct interaction with viral proteins [85]. Moreover, the gene TRITD4Bv1G182160 codes for a disease resistance protein (TIR-NBS-LRR class), that can be a viral resistance gene, providing monogenic dominant resistance, as shown in plants for other similar disease resistance proteins [86]. Finally, TRITD4Bv1G182400 codes for a putative RNA exonuclease 4 (REX4), a class of exonucleases involved in cleaving single-stranded RNA and DNA. In yeast, one of these RNA exonucleases (Interferon-stimulated exonuclease gene 20 (ISG20)), plays a role in mediating interferon's antiviral activities [87].

4.5.2. Candidate genes for WSSMV resistance

The two regions associated to WSSMV response included different genes that can be putatively involved in this trait (Table 4).

Two 3-ketoacyl-CoA synthase (KCS) encoding genes (TRITD6Av1G204040, TRITD4Bv1G163160) are present. These enzymes are involved in the formation of cuticle, and in particular in the metabolism of very-long-chain fatty acids (VLCFAs), that are required in all plant cells for the synthesis of essential membrane lipids and also used as precursors of protective cuticular waxes and suberin in leaves, epidermis, and roots. This cuticular waxes can influence the movement of pathogens, including viruses or virus-vectors [88]. Interestingly, Zhang and coworkers found that fatty acid elongation metabolism genes, including the expression of KCSs, were significantly down-regulated in the leaves of pecan mosaic virus (PMV)-infected plants and suggested that the expressions of KCSs, as well as the biosynthesis of VLCFAs, are involved in response to resistance to PMV infection in pecans [89]. Expansins may play a similar role in influencing viral replication and movement [90,91]. Interestingly, the region on chromosome 4B contains six expansin-encoding genes (TRITD4Bv1G161710, TRITD4Bv1G161730, TRITD4Bv1G161750, TRITD4Bv1G161770, TRITD4Bv1G162510, TRITD4Bv1G162530, TRITD4Bv1G162540).

Another enzyme that is involved in a general hypersensitive response (HR)-related resistance is glutathione S-transferase (TRITD4Bv1G163000) [92]. The four genes TRITD6Av1G206160, TRITD6Av1G206170, TRITD6Av1G206210, and TRITD6Av1G206620 code for PR-1 proteins that are known to be involved in the defence mechanism named pathogen-associated molecular patterns (PAMPs) [93]. Another interesting gene that could be involved in WSSMV response is TRITD4Bv1G162570, which sequence shows similarity with different proteins annotated as "inactive protein RESTRICTED TEV MOVEMENT 2-like" (*e.g.*, *Tr. aestivum* XP_044372927.1 and *Tr. dicoccoides* XP_037428118.1). In Arabidopsis, the Restricted Tobacco-Etch Virus Movement genes are well characterized potyvirus resistance genes, since they limit long-distance transport [94]. Tobacco-etch virus and WSSMV belong to the same virus family of Potyviridae, therefore it is possible that the gene TRITD4Bv1G162570 could similarly limit long-distance transport of WSSMV in wheat. Finally, TRITD4Bv1G162950 codes for an ATP-dependent RNA helicase, a possible susceptibility factor since it can promote viral replication and translation [95].

To our knowledge, only one specific gene has been identified and characterized in wheat for the resistance to bymoviruses [96,97]. A susceptibility factor to plant viruses that belongs to the conserved *PROTEIN DISULFIDE ISOMERASE* (*PDI*) gene family was identified first in barley [98] and then in wheat [91]. In barley, loss-of-function *HvPDIL5–1* alleles at the recessive *RESISTANCE TO YELLOW MOSAIC DISEASE 11 (rym11)* resistance locus conferred broad-spectrum resistance to multiple strains of Bymoviruses and could therefore play a central role in durable virus resistance breeding in barley [98]. A similar result was obtained in hexaploid wheat lines where the three homoeologous genes that are orthologues of *HvPDIL5–1* were mutated through CRISPR/Cas9: these lines showed resistance to the bymovirus wheat

Table 3
List of putative candidate genes involved in SBCMV resistance.

Trait	Gene ID	Annotation	Chr	Putative function
SBCMV resistance	TRITD4Bv1G179760	RNA-binding protein	4B	Viral RNA degradation
	TRITD4Bv1G180460	Defensin	4B	direct interaction with the virus envelope
	TRITD4Bv1G180870	HR-like lesion-inducing protein-related protein	4B	involvement in hypersensitive response
	TRITD4Bv1G181320	Appr−1-p processing enzyme family protein	4B	involvement in viral RNA replication
	TRITD4Bv1G182080	DET1-and DDB1-associated protein 1	4B	direct interaction with viral proteins
	TRITD4Bv1G182160	disease resistance protein (TIR-NBS-LRR class)	4B	viral resistance gene, providing monogenic dominant resistance
	TRITD4Bv1G182400	RNA exonuclease 4	4B	involved in cleaving single-stranded viral RNA

Table 4
List of putative candidate genes involved in WSSMV resistance.

Trait	Gene ID	Annotation	Chr	Putative function
WSSMV resistance	TRITD6Av1G204040	3-ketoacyl-CoA synthase	6A	cuticle formation
	TRITD6Av1G206160, TRITD6Av1G206170, TRITD6Av1G206210, TRITD6Av1G206620	Pathogenesis-related protein 1	6A	involvement in pathogen-associated molecular patterns (PAMPs)
	TRITD4Bv1G161710, TRITD4Bv1G161730, TRITD4Bv1G161750, TRITD4Bv1G161770, TRITD4Bv1G162510, TRITD4Bv1G162530, TRITD4Bv1G162540	Expansin	4B	involvement in viral replication and movement (susceptibility factor)
	TRITD4Bv1G162570	Heat shock family protein	4B	involvement in viral movement as putative RESTRICTED TEV MOVEMENT 2-like protein
	TRITD4Bv1G162950	ATP-dependent RNA helicase	4B	promoting viral replication and translation (susceptibility factor)
	TRITD4Bv1G163000	Glutathione S-transferase	4B	Maintaining resistance/preventing spread of localized cell death (oxidative stress)
	TRITD4Bv1G163160	3-ketoacyl-CoA synthase	4B	cuticle formation

yellow mosaic virus (WYMV) [91].

Another important class of recessive genes for resistance to viruses, including bymoviruses, is constituted by eukaryotic translation initiation factors (eIF), which are widely distributed in plant species [99]. The eIF4E and eIF4G are essential plant components for many RNA viruses to achieve their infectious cycle. Hence, the absence or alteration of eIF4E or eIF4G caused by point mutations or deletions can lead to total or partial resistance to viruses. Barley carries two genes, *rym4* and *rym5*, which code for two eIF4E proteins and confer recessive resistance to various bymoviruses [100]. In the chromosomal regions surrounding the QTNs associated to WSSMV response no gene encoding PDI or eIF was detected.

5. Conclusion

In the present work, we tested a panel of 212 durum wheat landraces and cultivars for the response to two soil-borne viruses, the furovirus SBCMV and the bymovirus WSSMV, using a specific experimental set up. The analysis of the virome present in the used soil, that excluded the presence of soil-borne viruses other than SBCMV and WSSMV, gave us confidence to deal with a known viral inoculum without the possibility of overlooking other viral diseases that could influence the observed phenotypes. The notable temperature and day length variations during the four months of the experiment certainly represent a condition closer to the real natural environment where plants grow through the winter season. We evaluated the symptomatology through visual scoring and measured the viral load present in leaf tissues through qRT-PCR. The 212 genotypes exhibited different responses to the two viruses: SBCMV accumulated in the tested genotypes with very diversified viral loads, whereas WSSMV infected all the durum wheat genotypes tested, with high viral loads in many of them. We identified 23 genotypes with low viral loads of both viruses and reduced symptoms that could be of particular interest for breeders aiming at new durum wheat varieties with resistance to both viruses. To our knowledge, this is the first work that presents a comprehensive analysis of a panel of wheat landraces for the response to these two soil-borne viruses simultaneously. Future efforts should pay more attention to WSSMV, the importance of which has been largely underestimated, as demonstrated by the fact that most of the durum wheat modern cultivars do not show resistance traits to it. Our association study allowed to identify new genomic regions possibly associated to resistance to SBCMV or WSSMV, as well as possible candidate genes involved in the two traits. These candidate genes constitute a basis for further specific studies aimed at identifying the genes that contribute to resistance in wheat. Although this analysis needs to be validated through further experiments, we envisage that the data obtained in this study will be a valuable resource for future research addressed to depict the resistance to these two soil-borne viruses in wheat.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2025.100485.

Data availability

Data will be made available on request.

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