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The Plant Genome

ORIGINAL RESEARCH

Grass pea natural variation reveals oligogenic resistance to *Fusarium oxysporum* f. sp. *pisi*

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Abstract

Grass pea (*Lathyrus sativus* L.) is an annual legume species, phylogenetically close to pea (*Pisum sativum* L.), that may be infected by *Fusarium oxysporum* f. sp. *pisi* (*Fop*), the causal agent of fusarium wilt in peas with vast worldwide yield losses. A range of responses varying from high resistance to susceptibility to this pathogen has been reported in grass pea germplasm. Nevertheless, the genetic basis of that diversity of responses is still unknown, hampering its breeding exploitation. To identify genomic regions controlling grass pea resistance to fusarium wilt, a genome-wide association study approach was applied on a grass pea worldwide collection of accessions inoculated with *Fop* race 2. Disease responses were scored in this collection that was also subjected to high-throughput based single nucleotide polymorphisms (SNP) screening through genotyping-by-sequencing. A total of 5,651 high-quality SNPs were considered for association mapping analysis, performed using mixed linear models accounting for population structure. Because of the absence

Abbreviations: AUDPC, area under disease progress curve; BGI, Beijing Genomic Institute; BLUE, best linear unbiased estimate; dai, day after infection; DI30, disease intensity at 30 dai; DIr, disease progress rate; ff. spp., formae speciales; Fo, Fusarium oxysporum; *Fop*, *Fo* f. sp. *pisi*; GWAS, genome-wide association study; hai, hour after infection; LD, linkage disequilibrium; LG, linkage group; PCR, polymerase chain reaction; qPCR, quantitative PCR; Q-Q, quantile-quantile; RCBD, randomized complete block design; RT-qPCR, Reverse Transcribed quantitative (real-time) PCR; SNP, single nucleotide polymorphism.

of a fully assembled grass pea reference genome, SNP markers' genomic positions were retrieved from the pea's reference genome v1a. In total, 17 genomic regions were associated with three fusarium wilt response traits in grass pea, anticipating an oligogenic control. Seven of these regions were located on pea chromosomes 1, 6, and 7. The candidate genes underlying these regions were putatively involved in secondary and amino acid metabolism, RNA (regulation of transcription), transport, and development. This study revealed important fusarium wilt resistance favorable grass pea SNP alleles, allowing the development of molecular tools for precision disease resistance breeding.

1 | INTRODUCTION

Grass pea (*Lathyrus sativus* L.) is an annual legume crop important for animal and human consumption in several rainfed marginal regions of the world (Lambein et al., 2019). In what concerns human consumption, grass pea may be consumed dried, cooked in stews, as well as uncooked as a green snack. This habit of eating grass pea in the green state is particularly widespread in Spain, but also in India, Bangladesh, and Pakistan, where the green pods and immature seeds are appreciated for the sweet and tasty flavor and valorized for this feature (Campbell, 1997; Peña-Chocarro & Peña, 1999). Grass pea accessions can be divided into two ecotypes: one with accessions with light and large seeds, typically from Europe and North Africa, and the other with accessions with dark and small seeds, mainly from Asia and Ethiopia (Hanbury et al., 1999; Przybylska et al., 2000). Normally explored for their exceptional resistance to drought, grass pea is also known for their resistance to legume pests and diseases (Campbell, 1997). Being a diploid species and predominantly self-pollinated, grass pea is also characterized by a large genome size (approximately 6.3 Gb) (Emmrich et al., 2020). Because of its genome complexity, together with the lack of economic relevance in developed countries, few studies have been performed to clarify grass pea genetic control of important traits such as disease resistance, when compared with other legume crops. This has hampered a more efficient development of resistant cultivars.

Among grass pea natural variation, a diversity of phenotypic responses has been found to pathogens commonly infecting pea (*Pisum sativum* L.), as *Uromyces pisi* (pea rust causal agent), *Erysiphe pisi* (pea powdery mildew causal agent) (Almeida et al., 2014; Vaz Patto et al., 2006), and more recently also *Fusarium oxysporum* f. sp. *pisi* (pea fusarium wilt causal agent) (Sampaio et al., 2021a).

Fusarium wilt, caused by the soil-borne pathogen *Fusarium oxysporum* (*Fo*) promotes huge worldwide yield losses in several plant species, including legumes (Sampaio et al., 2020). Considered a specialist pathogen, *Fo* is characterized by different *formae speciales* (ff. spp.) and races identified based on their preferential host species (Di Pietro et al., 2003). The genetic basis of resistance against fusarium wilt was unraveled for some of the *Fo* ff. spp. infecting major legume crops, such as chickpea (*Cicer arietinum* L.), common bean (*Phaseolus vulgaris* L.), and pea (Sampaio et al., 2020). Currently, the four different races identified as infecting pea are known as *Fo* f. sp. *pisi* (*Fop*), being *Fop* races 1 and 2 the most frequent worldwide (Infantino et al., 2006). Pea resistance to *Fop* race 1 was described as monogenic, with a major resistance gene, *Fw* (Grajal-Martìn & Muehlbauer, 2002; McClendon et al., 2002) located on chromosome 5 (Kreplak et al., 2019). Although resistance to *Fop* race 2 was also initially reported as monogenic (Infantino et al., 2006), further analysis of a segregating pea recombinant inbred line population revealed an oligogenic resistance depending on three resistance genes (McPhee et al., 2012). More specifically, a major gene, *Fnw4.1*, located on pea chromosome 4, and two minor resistance genes, *Fnw3.1* and *Fnw3.2*, both on pea chromosome 5 (McPhee et al., 2012).

Besides pea, *Fop* race 2 was also virulent to the close phylogenetic relative, grass pea, although with lower virulence when compared with pea (Sampaio, et al., 2021a). During a phenotypic screening of a grass pea worldwide collection of accessions to *Fop* race 2 infection, a wide range of responses was detected. Different resistance mechanisms within grass pea accessions contributed to the natural variation of phenotypes observed (Sampaio et al., 2021b). Nevertheless, the genetic basis controlling that diversity of responses is still unknown.

In precision breeding, the use of molecular markers tightly associated with the genetic factors controlling resistance allows a more efficient, faster, and affordable selection approach compared with the conventional plant breeding programs on the development of resistant cultivars (Rubiales et al., 2015; Sampaio et al., 2020). However, for a precision breeding application, understanding the genetic basis of resistance is essential.

Genome-wide association studies (GWAS) are powerful approaches to identify genomic regions responsible for certain traits using the naturally occurring genetic diversity (Korte & Farlow, 2013). In legumes with a fully assembled reference genome, such as common bean and cowpea [*Vigna unguiculata* (L.) Walp.], GWAS was successfully used to unravel the genetic control of Fusarium wilt resistance, allowing the identification of several candidate resistant genes and confirming an oligogenic control in both species (Leitão et al., 2020; Wu et al., 2015). However, in species where a reference genome is still not available or not fully assembled, such as in grass pea (Emmrich et al., 2020), the interpretation of GWAS results will be more challenging. The recently released pea reference genome v1a (Kreplak et al., 2019) was already used, through comparative mapping, to propose candidate resistance genes to *Erysiphe pisi* and *E. trifolii* within QTL regions located originally in the related *Lathyrus cicera* (Santos et al., 2020). In addition, pea genome was also used to enable comparative mapping of the powdery mildew susceptibility gene *MLO1* (*Mildew Locus O 1*) in *L. sativus* (Santos et al., 2021), further highlighting its value as a promising tool to facilitate genetic studies in *Lathyrus* spp.

The present study aims to clarify the genetic control of grass pea resistance to *Fop* race 2 through GWAS. For that, a high-throughput single nucleotide polymorphism (SNP) screening of a worldwide collection of grass pea accessions, previously evaluated for the response to *Fop* race 2 infection (Sampaio et al., 2021b), was conducted and SNP–trait associations analyzed. To the best of our knowledge, this is the first time a GWAS has been applied to grass pea and, in particular, for grass pea–fusarium wilt resistance genetic analysis.

Core Ideas

- The first grass pea disease resistance genome-wide association study was conducted.
- Grass pea revealed an oligogenic control to fusarium wilt.
- 17 significantly fusarium wilt resistance associated SNPs were detected.
- Candidate genes were involved in secondary/amino acid metabolism, RNA, transport, and development.

2 | MATERIAL AND METHODS

2.1 | Phenotypic screening

The phenotypic data set used in the present association analysis was retrieved from Sampaio et al. (2021b). Data collection will be briefly explained. For further details, please refer to Sampaio et al. (2021b).

2.1.1 | Fungal material

The *Fop* race 2 strain R2F42 used in this study, was provided by Dr. W. Chen (USDA-ARS, Pullman, USA) and stored as microconidial suspension at −80 ˚C in 30% glycerol. For microconidia production, cultures were grown in potato dextrose broth (Sigma-Aldrich) at 28 ˚C, in a shake culture at 170 rpm for 3/4 d (Di Pietro & Roncero, 1998).

2.1.2 | Plant material and growth conditions

Data from 161 grass pea accessions belonging to the germplasm collection characterized in Sampaio et al. (2021b) was used in the present GWAS. From these 161 accessions, 79 had a European geographical origin, 54 Asian, 13 Ethiopian, 9 Northern African, 1 Brazilian, and 5 with unknown origin. Of these 161 accessions, 78 presented light and 83 dark seed color, 44 had large and 117 had small seed size. Details in Supplemental Table S1. Seedlings were planted in individual pots containing sterile vermiculite and placed on a growth chamber at 26 ± 2 ˚C, 16 h (light)/8 h (dark) and 60% relative humidity.

2.1.3 | Inoculation and disease assessment

Because of growth chamber space constraints, the grass pea collection was mostly tested using an alpha-lattice experimental design (Patterson & Williams, 1976) (129 accessions), complemented with a smaller randomized complete block design (RCBD, in 32 accessions) (Sampaio et al., 2021b). Five grass pea accessions (PI220176, PI283582, PI283593, PI283596, and PI426880), characterized by an intermediate response to *Fop*, were repeatedly evaluated in all the blocks of the two designs, to control for block effects. Three independent inoculation experiments were conducted and three to five plants per accession were evaluated per experiment. Seven-day-old grass pea seedlings were inoculated following a root dip technique with trimmed roots and then replanted in pots under the same growth conditions.

For each plant, at every 3 d from the 7th to the 30th d after infection (dai), disease intensity (DI) was evaluated by calculating the percentage of yellowing leaves per total leaves (Figure 1). This data was used to calculate AUDPC (area under disease progress curve) and DIr (disease progress rate) (Sampaio et al., 2021b). In addition, maximum DI score observed at 30 dai (DI30), in %, was also recorded.

FIGURE 1 *Fusarium oxysporum* f. sp. *pisi* race 2 disease assessment in the grass pea collection. Numbers below each photograph represent their respective disease intensity (DI), estimated as the percentage of leaves showing symptoms

2.1.4 | Phenotypic data analysis

The alpha experimental design and RCBD data sets were analyzed separately. AUDPC data were subjected to a square root transformation in both data sets (RCBD and alpha design) to guarantee residuals normalization and variance stabilization (Sampaio et al., 2021b).

Using the linear mixed models described by Sampaio et al. (2021b), a restricted maximum likelihood procedure was conducted, after an independent quality control of each trait, to obtain the best linear unbiased estimates (BLUEs) for each accession and for both data sets separately. Due to high correlation, and similar values between the five grass pea accessions repeatedly evaluated in both experimental designs (Sampaio et al., 2021b), the two BLUEs vectors, from the two different designs, were merged into a single data set. The merged BLUE data set (Supplemental Table S2) was the input phenotypic data in the subsequent association mapping analysis.

All analyses were performed with Genstat software, 20th ed. (VSN).

2.2 | Genotypic screening

Total genomic DNA from one representative individual of each grass pea accession was extracted from young leaves using the DNeasy Plant Mini Kit (Qiagen), according to manufacturer's instructions. DNA quantification was performed in a Qubit 2.0 Fluorometer (Invitrogen) using Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). DNA purity was assessed by wavelength ratios measurement at 260:280 and 260:230 nm using a Nanodrop ND-2000C spectrophotometer (Thermo Fisher Scientific).

The extracted DNA samples were sent for SNP detection and calling to two high-throughput genotyping-by-sequencing (GBS) providers: Diversity Arrays Technology through DArT-Seq™ genotyping (Kilian et al., 2012) and BGI (Beijing Genomic Institute).

2.2.1 | Genotypic data analysis

2.2.1.1 SNP data acquisition improvement

To improve SNP data acquisition obtained from BGI Tech Solutions, a MOCK reference approach was used. In brief, the clean and demultiplexed reads were submitted to GBS SNP-Calling Reference Optional Pipeline version 4.0 (GBS-SNP-CROP) for sequence analysis and genotyping (Melo et al., 2016). The GBS-SNP-CROP was used for de novo SNP calling. The GBS specific mock reference was provided from the sample with the highest number of clean reads using GBS-SNP-CROP-4.pl script to cluster reads and assemble the mock reference. The mapping step was performed with GBS-SNP-CROP-5.pl script, where clean reads were mapped to the mock reference to produce standard alignment files using BWA-mem (Li, 2013) and SAMtools (Li et al., 2009). The SNP and genotype calling step was carried by GBS-SNP-CROP-7.pl script. Finally, the SNP genotyping matrix was converted into formats compatible with Tassel GUI (Glaubitz et al., 2014) for further analysis.

2.2.1.2 Quality control

Genotypic data quality control was performed by removing SNP markers and accessions with more than 25% missing data. SNP calls recorded as heterozygous were set as missing data. Markers with minor allele frequency smaller than 5% were removed.

2.2.1.3 SNP markers position

Because no fully assembled *L. sativus* reference genome is publicly available, SNP markers physical positions were assigned based on the pea reference genome v1a (Kreplak et al., 2019), the most phylogenetic closely related species to grass pea, with a better assembled sequenced genome. In addition, high synteny between grass pea recombinant inbred line high-density linkage map and the pea genomes was recently revealed (Santos et al., 2021). For that, a BLASTn alignment (E-value $< 1 \times 10^{-5}$; word size = 11) between grass pea markers sequence without the initial adapters

("TGCAG" for DArT-Seq™ and "CAGC" for BGI sequence) and the pea genome v1a was performed at KnowPulse web resource (https://knowpulse.usask.ca) (Sanderson et al., 2019) database.

2.2.1.4 Genetic structure analysis

A subset of 1,058 SNP was chosen by considering approximately 1 Mbp interval between SNPs within each pea chromosome (Supplemental Table S3). This subset was used on a model-based clustering method to infer genetic structure and define the number of genetic clusters in the dataset using the software STRUCTURE v2.3.4 (Pritchard et al., 2000). Thirty runs per each cluster (K) ranging from 1 to 11 were conducted on the Isabella computer cluster at the University of Zagreb, University Computing Center (SRCE), Croatia. Each run was comprised of a burn-in period of 20,000 steps, followed by 10^6 MCMC (Monte Carlo Markov Chain) replicates assuming an admixture model and correlated allele frequencies. The ΔK values were calculated using STRUCTURE HARVESTER v0.6.94 (Earl & vonHoldt, 2012). Runs were clustered and averaged using CLUMPAK (Kopelman et al., 2015). Accessions were assigned to a particular cluster (A, B, or C) if their proportion of membership (Q) in that cluster was greater than 75%, whereas those with a Q value below 75% were considered to be of "mixed origin" and were designated AM, BM, or CM.

In addition, the same molecular data set was used to calculate principal components as a complementary approach to access population structure and to calculate a kinship matrix to estimate pairwise genetic relatedness among the grass pea accessions, as implemented in Genstat software. The obtained SNP-based genetic structure was visually compared with seed color, size, and geographical origin grouping.

2.3 | Association mapping analysis

Genome-wide association studies to detect grass pea AUDPC, DI30, and DIr associated SNPs were conducted with Genstat software in the mixed model framework, fitting markers as fixed and accessions as random terms using restricted maximum likelihood (Malosetti et al., 2007).

Grass pea adjusted means (BLUES) of the three traits were tested for association with the SNP markers fitting the quality control parameters applied. To detect marker–trait associations, three different models were tested: a naïve model [$Phenotype = SNP + Error$], which does not account for population structure or family relatedness; a model accounting for population structure (Q), using 15 principal components from the principal component analysis [$Phenotype = Q + SNP + Error$]; and a model accounting for familial relatedness (K), using a kinship matrix K [$Phenotype = SNP + Accession + Error$], with $Accession$ random effects structured following a kinship matrix K (Malosetti et al., 2007).

Inflation factor values near 1 and quantile-quantile (Q-Q) plots of the respective p-values with lower deviations from the expected uniform distribution under the null hypothesis were the considered parameters to select the best model accounting for genetic structure/relatedness among accessions. The observed association p-values ($-\log_{10}$ scale) for each SNP were plotted against their chromosomal pea position generating Manhattan plots. A threshold of $-\log_{10}(p) = 4$, controlling background noise, was used to detect significant marker–trait associations. As in other association studies with similar or slightly smaller panel sizes (Alves et al., 2019, 2020; Leitão et al., 2020, 2021), the threshold was selected to remove the background noise of the Manhattan plot, allowing at the same time the detection of potentially interesting regions, which would be lost using a more conservative type as Bonferroni-corrected threshold of significance. The effect of the minor frequency SNP allele was estimated in relation to the most frequent reference allele.

2.4 | Allelic variant frequency and cluster analysis based on the SNPs associated with the traits of interest

Because the grass pea accessions under study were considered very homozygous due to several generations of single seed descent, favorable allele frequencies were calculated by counting the number of accessions with a given trait (dark, light, small, or large) that had the favorable allele and divided by the total number of accessions having the same trait.

For the grass pea accessions cluster analysis based on the associated SNPs, seventeen SNPs associated with the traits of interest (see Results section) were used to construct the genetic distance matrix between pairs of grass pea accessions using the proportion-of-shared-alleles distance (D_{PSA}; Bowcock et al., 1994) in MICROSTAT (Minch et al., 1997). The unweighted pair group method with arithmetic mean (UPGMA) tree was constructed using PHYLIP v3.6b (Felsenstein, 2004).

2.5 | Local linkage disequilibrium and putative candidate genes

Linkage disequilibrium (LD) was calculated per chromosome as a measure of the recombination history, estimated by the squared coefficient of correlation between markers pairs, r^2, after correction for population structure using the principal component scores from Eigenanalysis, as implemented in Genstat software. For this calculation, all grass pea markers with an assigned position on pea reference genome v1a were used. Linkage disequilibrium decay per chromosome was visualized by plotting r^2 against the physical mapping

distance in Mbp. To define a genomic window where to search for candidate genes, the presence of adjacent SNP markers in LD with the ones identified as significantly associated with the trait was investigated. The r^2 of the neighboring SNPs was inspected considering, right and left to the associated SNP, a strict LD decay threshold of $r^2 > 0.2$.

Genes containing a significant associated SNP [$-\log_{10}(p) \geq 4$] or a SNP in LD with the significant associated SNP for AUDPC, DI30, and DIr were considered putative candidate genes for the disease response traits analyzed. Pea genome (Kreplak et al., 2019) was used as the reference genome for candidate genes investigation. Annotation of the genes under the identified genomic regions was given by the JBrowse tool at https://urgi.versailles.inra.fr/Species/Pisum. Candidate genes' functional characterization was obtained using the MapMan web tools and Mercator4 v2.0 (https://www.plabipd.de/portal/mercator4) (Schwacke et al., 2019). Cytoscape software, version 3.8.2 (Shannon et al., 2003) was used to visualize molecular interaction networks occurring between the traits.

2.6 | Comparative mapping of pea-unmapped associated SNPs

Associated SNPs without a known position in *P. sativum* reference genome v1a were aligned against *Lens culinaris* CDC Redberry genome v1.2 and *Medicago truncatula* genome Mtv4.0 (Tang et al., 2014), using the BLASTn tool (E-value $< 1 \times 10^{-5}$) at KnowPulse web resource (https://knowpulse.usask.ca) (Sanderson et al., 2019) database.

Santos et al. (2021) syntenic studies between *L. sativus* and *L. culinaris* and/or *M. truncatula*, were considered to attribute putative *L. sativus* linkage group (LG) locations of the pea-unmapped associated SNPs through comparative mapping.

2.7 | Candidate genes relative expression analysis by Reverse Transcribed quantitative (real-time) PCR

2.7.1 | Plant material, RNA extraction, and cDNA synthesis

Three highly *Fop* resistant accessions (low AUDPC, DI30, and DIr: BGE19777, PI283593, and SITNICA) and three highly susceptible (high AUDPC, DI30, and DIr: PI195998, PI257589, and PI358601) were selected for the candidate genes' relative expression analysis based on responses characterized by Sampaio et al. (2021b). Three individual plants per accession (biological replicates) were used for each of two treatments (inoculated/noninoculated) and plant inoculation was conducted as previously described. Four time points after infection were defined to collect root samples from both treatments: 24 and 48 h after infection (hai) and 4 and 7 dai. Plants were removed from the pots and washed under tap water to remove vermiculite from the roots. Root samples were then collected, immediately frozen in liquid nitrogen, and stored at −80 ˚C.

For total RNA extraction, frozen roots were ground to a fine powder in liquid nitrogen using a mortar and a pestle. Total RNA was isolated from each plant sample and treatment separately, using the GeneJET Plant RNA Purification Kit (Thermo Scientific) following the manufacturer's protocol. Isolated RNA was treated with TURBO DNase (Invitrogen by ThermoFisher Scientific) according to the manufacturer's protocol. RNA quantification was conducted in a Qubit 2.0 Fluorometer (Invitrogen) with a Qubit RNA BR Assay Kit (Thermo Fisher Scientific). RNA purity was assessed by wavelength ratios measurement at 260:280 and 260:230 nm using a Nanodrop ND-2000C spectrophotometer (Thermo Fisher Scientific). RNA integrity was also checked by electrophoresis in a 1% agarose gel stained with SYBR Safe (Thermo Fisher Scientific).

cDNA was synthesized from 1 μg of total RNA from each sample using the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer's protocol.

2.7.2 | Primer design

Specific primers were designed for all the potential candidate genes using the gene sequence obtained from the JBrowse tool at https://urgi.versailles.inra.fr/Species/Pisum using the Primer3Plus online tool (https://primer3plus.com). Primer specificity was checked using the Primer-BLAST NCBI tool considering the *M. truncatula* genome. Primer design parameters were defined using the default setting for qPCR optimal conditions. Primers were designed in the 3′ intra-exonic region and synthesized by STABVida (Supplemental Table S4).

2.7.3 | Reverse Transcribed quantitative (real-time) PCR

Relative expression of target genes among the selected resistant and susceptible grass pea accessions was determined by Reverse Transcribed quantitative (real-time) PCR (RT-qPCR). As reference genes, β-tubulin, photosystem I P700 apoprotein A2, and γ-tubulin, previously described as reference genes in *Lathyrus* spp. (Almeida et al., 2014; Santos et al., 2018), were tested. Using geNorm and NormFinder software packages, from GenEx v.5 software (MultiD), the first two were selected as reference genes for the following analysis. RT-qPCR was performed for each of the three

biological replicates per accession (resistant and susceptible) and per treatment (noninoculated and *Fop* inoculated) at each time point (24 and 48 hai, 4 and 7 dai). PCR reactions were carried out in a PikoReal Real-Time PCR System (Thermo Fisher Scientific) using PerfeCTa SYBR Green SuperMix (Quantabio) following the described conditions: 5 min at 95 ˚C for initial denaturation, 40 cycles of denaturation at 95 ˚C for 10 s and 60 ˚C for 30 s. For each reaction, a melting curve (dissociation stage) was performed to detect nonspecific PCR products or contaminants. A nontemplate control without cDNA was also included for each primer mix to detect possible contaminations.

The relative expression values of each gene were normalized per accession to the respective 24 hai control sample and to the two reference genes following the Pfaffl method (Pfaffl, 2001). A two-way ANOVA was conducted to investigate differences between treatments and time points per candidate gene and accession. Post-hoc Tukey multiple comparison tests were used for means comparison at $p \leq .05$.

3 | RESULTS

3.1 | Genotypic screening

High-throughput SNP screening of the grass pea collection through DArT-Seq™ genotyping platform resulted in the detection of 15,818 SNPs. From these, only 3,496 (22%) passed the quality control criteria. Moreover, after the improvement of the original SNPs scoring by BGI (detecting 206 SNPs) using a MOCK reference approach, a total of 11,058 SNPs were detected, of which 2,155 SNPs (19.5%) passed the quality control criteria. Together these two GBS approaches yielded 5,651 high-quality SNPs that were used for the association mapping analysis. No common SNPs have been detected between the two GBS approaches. SNP markers list and the marker scores per accession are available in Supplemental Table S5.

From the 5,651 SNP markers, 3,180 presented a single hit on pea chromosomes, whereas the remaining 2,471 were considered unmapped due to lack of alignment with the pea genome or due to the presence of more than a single hit with the same E-value. The number of mapped markers per chromosome ranged from 291 SNP markers in chromosome 3 to 624 in chromosome 5.

3.2 | Association panel genetic structure

The STRUCTURE analysis, based on the subset of 1,058 SNP markers (Supplemental Table S3), identified $K = 2$ as the most likely number of genetic clusters (highest $\Delta K = 28{,}540.08$), followed by $K = 3$ ($\Delta K = 1{,}865.93$). At $K = 2$, most large/light seeded, large/dark seeded, and small/light seeded accessions belonged to cluster A, while cluster B consisted of small/dark seeded accessions that were predominantly of Asian origin (Figure 2). At $K = 3$, small/dark seeded accessions of Ethiopian origin and partly of Asian origin, which belonged to cluster A at $K = 2$, were assigned to the newly formed cluster C (Supplemental Table S6).

Based on the same subset of markers, principal components were also calculated to access the population structure of the grass pea collection. By visually comparing this structure with different seed grouping characteristics, a stronger clustering of lighter and larger seed accessions, mainly from Europe and North Africa was observed, while darker and smaller seed accessions were dispersed around the principal component analysis biplot (Figure 3).

3.3 | Marker–trait associations

Due to the existence of a clear genetic structure within the association panel, which can lead to the identification of false–positive associations, GWAS was performed using a linear mixed model accounting for the genetic structure, by considering either population structure (Eigenanalysis) or kinship relationship (K matrix).

The best model accounting for the grass pea collection genetic structure, by presenting an inflation value closer to 1 (Supplemental Table S7) and Q-Q plots showing less p-values deviating from the expected uniform distribution that holds under the null hypothesis (Supplemental Figure S1), was the linear mixed model accounting for the population structure (Eigenanalysis). This was the selected model and the results reported hereafter were obtained from this model. Manhattan plots for the discarded models (kinship and naïve) are available in Supplemental Figure S2.

For the three disease response traits analyzed (AUDPC, DI30, and DIr), previously identified as phenotypically highly correlated (Sampaio et al., 2021b), significantly associated SNPs were detected. In total, 17 SNPs were associated with at least one of the traits of interest using a threshold of $-\log_{10}(p) = 4$. Of these, 13 were commonly associated with all the traits measured. Seven of the associated SNPs had a known position on the pea genome, being located on gene coding regions of chromosomes 1, 6, and 7. The other 10 SNPs associated with resistance against fusarium wilt were unmapped (Figure 4 and Table 1).

From the 10 SNP markers with unknown position on pea chromosomes, only two associated SNPs, SNP3824 and SNP5205, revealed a physical position on *L. culinaris* (CDC Redberry genome v1.2) and/or *M. truncatula* (Mtv4.0) chromosomes. SNP3824 was located on *L. culinaris* chromosome 2, chromosome with high synteny with *L. sativus* LG I (Santos

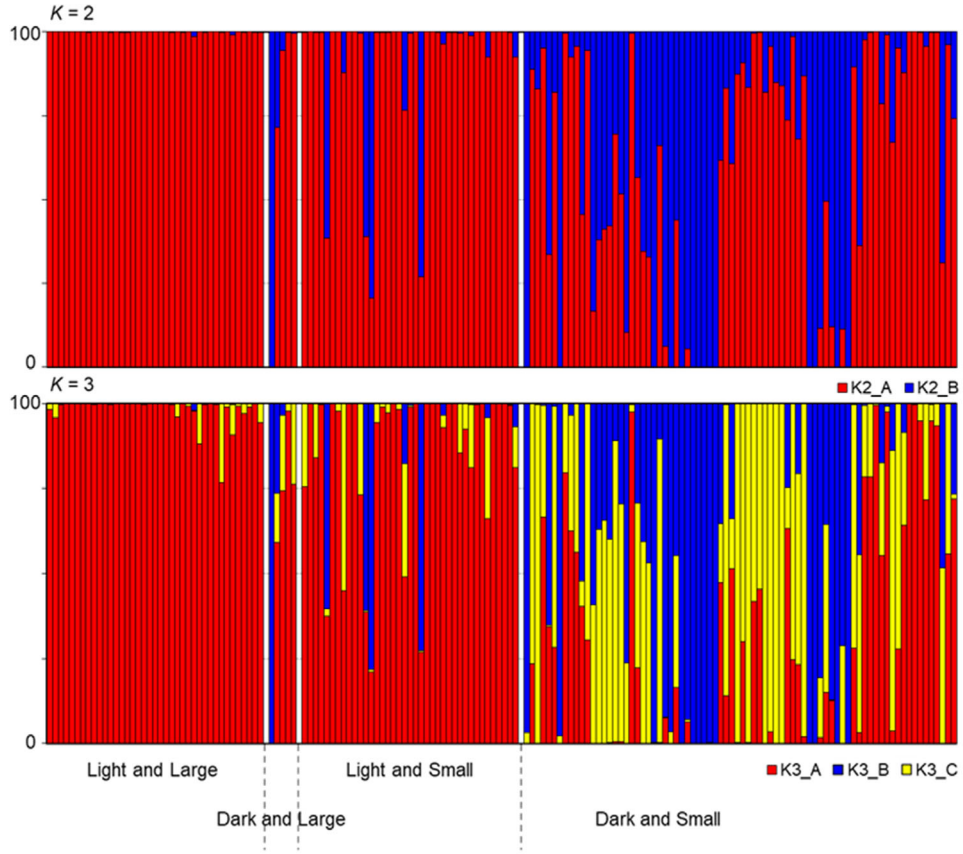

FIGURE 2 Genetic structure of the worldwide grass pea collection using STRUCTURE at $K = 2$ and $K = 3$. Accessions are grouped according to seed color (dark or light) and size (large or small). Each accession is represented by a column, and the color corresponds to the proportions of membership in each genetic cluster

et al., 2021), while SNP5205 was placed on *L. culinaris* chromosome 7 and *M. truncatula* chromosome 8, chromosomes characterized by high synteny with *L. sativus* LG IV (Santos et al., 2021). Grass pea LG I and LG IV are on the other hand mainly syntenic with pea chromosomes 1 and 4, respectively (Santos et al., 2021).

A positive effect of the variant allele in relation to the most frequent allele was observed in all the associated SNP, representing for the variant allele, an increase in *Fop* susceptibility (Table 1). For all the traits, each identified SNP–trait association explained only a part of the observed phenotypic variance. The associated SNP that explained the biggest portion of genotypic variance was SNP3907, simultaneously associated with the three traits and explaining 24.5% of AUDPC, 26.9% of DI30, and 29.1% of DIr variance (Table 1).

3.4 | Allelic variant frequency and cluster analysis based on the SNPs associated with the traits of interest

Favorable alleles of the associated SNP, responsible for an increase in resistance, were mainly the most frequent in both seed color (light and dark) and seed size (large and small) groups of our grass pea collection, with higher frequency in the lighter and larger seed accessions group (Figure 5). Although allele frequencies for lighter and larger seeds used to match, as well as smaller and darker seeds, the frequency differences between seed sizes tended to be higher than differences between seed colors. The only exceptions to this pattern were SNP2530, SNP2540, and SNP3824, where almost no favorable allele frequency differences between seed color and seed size groups were detected.

According to the accessions' geographical origin, favorable alleles were more frequent in European, Northern African, and Brazilian accessions, while Asian and Ethiopian accessions were shown to have a lower frequency of the allele responsible for conferring resistance (Figure 6). A slightly different pattern was observed for SNP3907, the SNP with the biggest portion of genotypic variance explained, and for SNP2530.

When focusing on grass pea accessions disease response ranking, for most of the associated SNPs, favorable alleles, conferring resistance, were no longer present in accessions showing an AUDPC square-root-transformed mean value ≥35, a DI30 mean value ≥80%, and a DIr mean value ≥3.0 (Supplemental Figure S3).

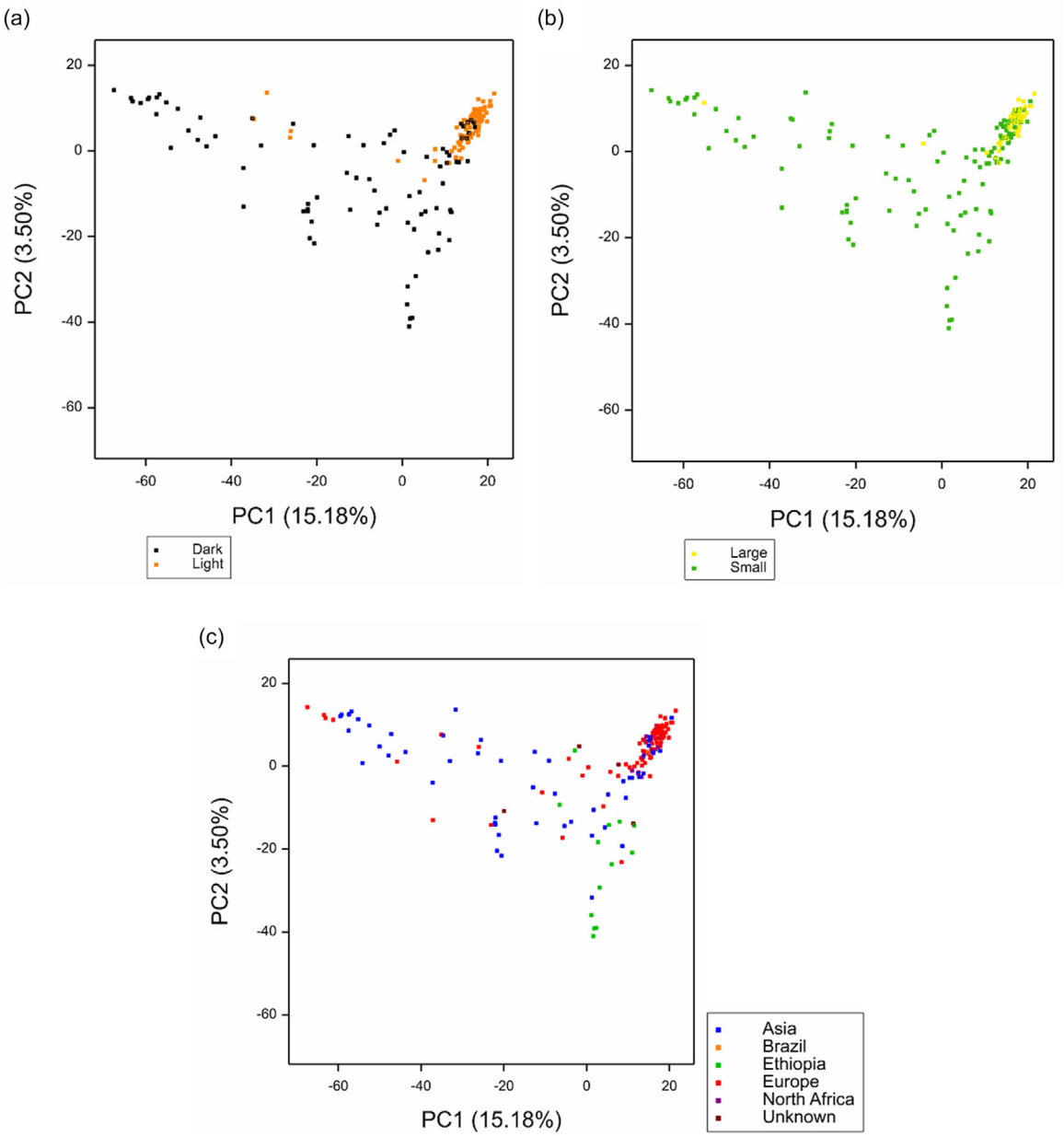

FIGURE 3 First two component scores from Eigenanalysis using 1,058 single nucleotide polymorphisms (SNPs) in 161 grass pea accessions organized according to (a) seed color, (b) seed size, and (c) geographical origin. The variance explained by each principal component is presented on the axis heading

Cluster analysis based on 17 SNPs associated with the traits of interest showed a clear separation of the grass pea accessions originating from Ethiopia, characterized by darker and smaller seeds and belonging to cluster C at $K = 3$ (Supplemental Figure S4).

3.5 | Candidate genes

The location of the SNPs significantly associated with fusarium wilt response traits was used to search for putative candidate genes in the pea genome v1a. Candidate genes could only be proposed for mapped SNPs. From the seven SNP-trait associations with a known pea map position, five candidate genes were proposed, since three of the seven significantly associated SNPs were mapped into the same gene. In this way, the strongest associated SNP, SNP2428, was located within the same candidate gene (*Psat6g142560*) as SNP2424 and SNP2427, a gene related with amino acid metabolism encoding a threonine aldolase. SNP2530, previously highlighted for the frequency of favorable allele among accessions with a different geographical origin, was located within *Psat6g185920*, a gene involved in the regulation of transcription. Moreover, SNP3540, highlighted due to the frequency of the favorable

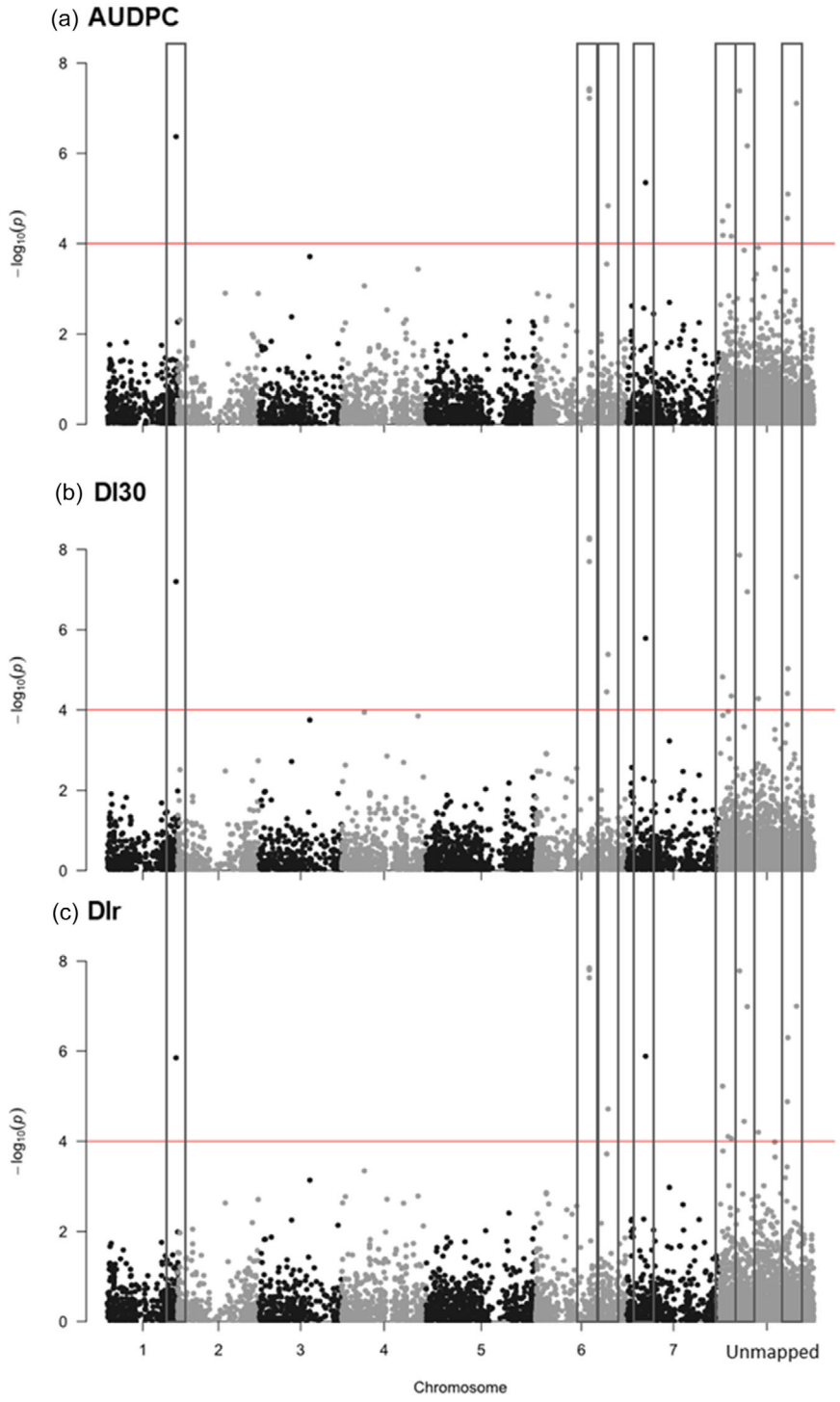

FIGURE 4 Manhattan plots depicting genome-wide association results for (a) area under disease progress curve (AUDPC), (b) disease intensity at 30 d after infection (DI30), and (c) disease progress rate (DIr) using 161 grass pea accessions inoculated with *Fusarium oxysporum* f. sp. *pisi* (*Fop*). The *y*-axis constitutes the $-\log_{10}(p)$ of 5,651 SNP markers, and the *x*-axis exhibits their chromosome position on the pea reference genome. The red horizontal line represents the threshold $-\log_{10}(p) = 4$. The columns highlight the genomic regions with commonly associated single nucleotide polymorphisms (SNPs) for the three traits

allele among accessions with different seed color and size, was located within *Psat6g189280*, a gene involved in development. SNP0375 and SNP2797 were each located within a candidate gene, respectively within *Psat1g212920*, a gene involved in transport, and *Psat7g059960*, a gene involved in secondary metabolism. Details on candidate gene function are described in Table 2.

Most candidate genes proposed were common to the three analyzed disease response traits, the only exception being, *Psat6g185920*, the gene involved in RNA (transcription regulation) that was only associated with DI30 (Supplemental Figure S5).

The distance to which LD decayed to $r^2 = 0.2$ per chromosome was graphically estimated, and varied from 0.008 Mbp (on chromosomes 1, 3, 5, and 7) to 0.14 Mbp (on chromosome 6), with an average value of 0.043 Mbp. Supplemental Figure S6 shows the LD decay, measured as r^2 values versus marker distance, and shade plots per chromosome exhibit the corre-

TABLE 1 Single nucleotide polymorphism (SNP) associations ($-\log_{10}[p] \geq 4$) with AUDPC, DI30, and DIr, their position within pea chromosomes (or grass pea linkage group, LG), the effect of the allelic variant, and the proportion of genotypic variance explained by each SNP-trait association using 161 grass pea accessions inoculated with *Fusarium oxysporum* f. sp. *pisi* (*Fop*)

Marker name	Trait	Pea chromosome/ grass pea LG	Position (Mbp)	$-\log_{10}$ (*p*)	Ref allele	Variant allele	Freq[a]	Effect[b]	V_{QTL}/V_g [c] %
SNP0375	$AUDPC^{SR}$	1	362.716539	6.53	A	G	0.055	6.06	9.7
	DI30			7.20				14.33	11.4
	DIr			5.85				0.48	9.7
SNP2424	$AUDPC^{SR}$	6	282.733165	6.83	G	A	0.062	7.28	15.7
	DI30			7.70				17.27	18.5
	DIr			7.63				0.63	19.1
SNP2427	$AUDPC^{SR}$	6	282.733287	7.00	C	T	0.058	7.40	15.2
	DI30			8.25				17.99	18.9
	DIr			7.80				0.64	18.7
SNP2428	$AUDPC^{SR}$	6	282.733319	7.04	A	T	0.058	7.42	15.2
	DI30			8.29				18.04	18.9
	DIr			7.85				0.65	18.7
SNP2530	DI30	6	374.644821	4.45	G	A	0.110	8.01	6.7
SNP2540	$AUDPC^{SR}$	6	381.67895	4.38	T	C	0.103	4.15	8
	DI30			5.39				10.42	10.7
	DIr			4.72				0.36	9.8
SNP2797	$AUDPC^{SR}$	7	99.915993	5.05	G	A	0.104	4.14	8.1
	DI30			5.79				9.98	9.9
	DIr			5.89				0.37	10.6
SNP3254	$AUPC^{SR}$	Unmapped		4.90	T	A	0.083	5.45	11.5
	DI30			4.84				12.04	11.8
	DIr			5.10				0.45	12.9
SNP3401	$AUDPC^{SR}$	Unmapped		4.40	T	A	0.070	5.41	9.7
	DIr			4.07				0.42	9.6
SNP3486	DI30	Unmapped		4.37	G	A	0.120	8.40	7.9
SNP3701	$AUDPC^{SR}$	Unmapped		6.68	T	C	0.133	4.15	10.1
	DI30			7.64				10.02	12.3
	DIr			7.82				0.37	13.3
SNP3824	DIr	LG I (pea chromosome 1 by comparative mapping[d])		4.43	G	T	0.1167	0.28	6.8
SNP3907	$AUDPC^{SR}$	Unmapped		6.56	G	A	0.211	5.38	24.5
	DI30			6.89				12.32	26.9
	DIr			7.13				0.46	29.1
SNP4197	$AUDPC^{SR}$	Unmapped		4.03	T	C	0.268	3.32	10.9
	DI30			4.39				7.76	12.6
	DIr			4.23				0.28	12.6
SNP4964	$AUDPC^{SR}$	Unmapped		4.27	A	G	0.119	3.98	8.4
	DI30			4.40				9.09	9.2
	DIr			4.85				0.35	10.7
SNP4976	$AUDPC^{SR}$	Unmapped		5.46	G	A	0.103	4.19	8.2
	DI30			5.08				9.07	8.1
	DIr			6.26				0.37	10.6

(Continues)

TABLE 1 (Continued)

Marker name	Trait	Pea chromosome/ grass pea LG	Position (Mbp)	$-\log_{10}(p)$	Ref allele	Variant allele	Freq[a]	Effect[b]	V_{QTL}/V_g [c] %
SNP5205	AUDPC^{SR}	LG IV (pea chromosome 4 by comparative mapping[d])		6.62	T	C	0.059	7.00	13.7
	DI30			7.32				16.40	15.8
	DIr			7.13				0.59	16

Note. AUDPC, area under disease progress curve; DI30, disease intensity at 30 d after infection; DIr, disease progress rate; SR, AUDPC values square-root-transformed.
[a]Frequency of the variant allele.
[b]Effect of the variant allele.
[c]Proportion of genotypic variance explained by each SNP-trait association. $V_{QTL} = 2Freq(1\text{-}Freq)effect^2$ and V_g = genotypic variance calculated from the alpha design (Sampaio et al., 2021b).
[d]As described in Santos et al.(2021).

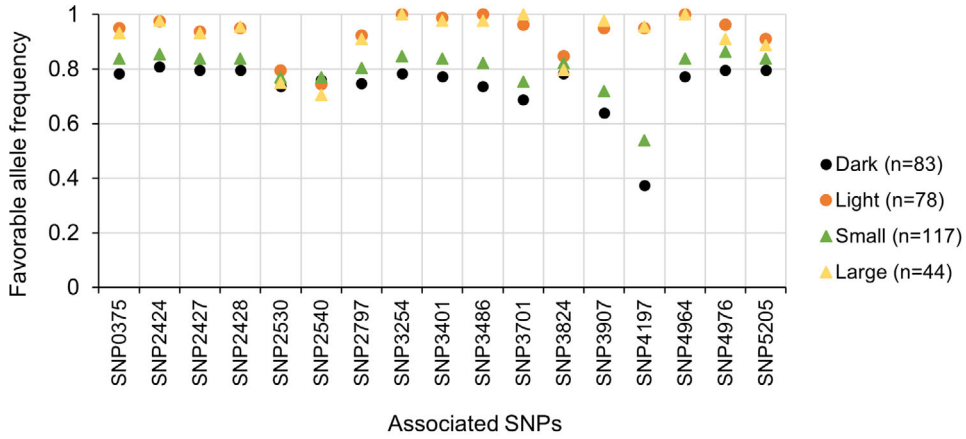

FIGURE 5 Frequency of the favorable (conferring resistance) allele of the 17 single nucleotide polymorphisms (SNPs) associated with fusarium wilt area under disease progress curve (AUDPC), disease intensity at 30 d after infection (DI30), and disease progress rate (DIr) based on the 161 grass pea accessions inoculated with *Fusarium oxysporum* f. sp. *pisi* (*Fop*) according to their morphological seed characteristics (color and size)

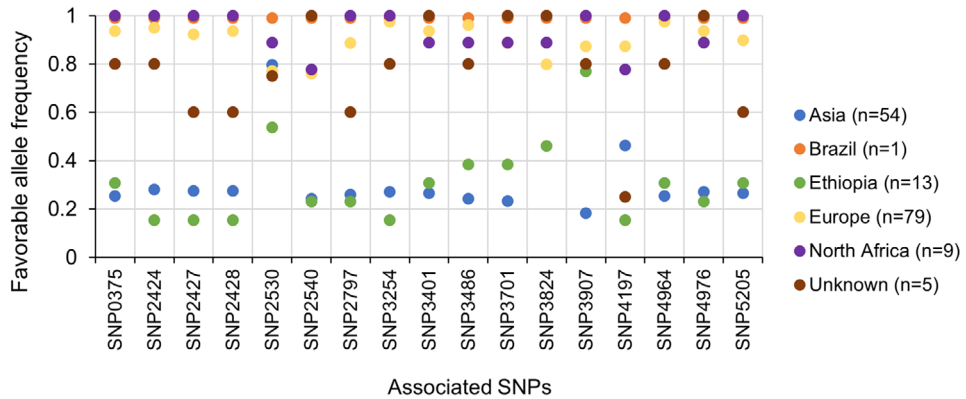

FIGURE 6 Frequency of the favorable (conferring resistance) allele of the 17 single nucleotide polymorphisms (SNPs) associated with fusarium wilt area under disease progress curve (AUDPC), disease intensity at 30 d after infection (DI30), and disease progress rate (DIr) based on the 161 grass pea accessions inoculated with *Fusarium oxysporum* f. sp. *pisi* (*Fop*) according to their geographical origin

TABLE 2 Candidate genes underlying the seven mapped-SNPs associated with grass pea AUDPC, DI30, and DIr after *Fusarium oxysporum* f. sp. *pisi* (*Fop*)infection

Marker	Trait	Associated candidate gene and location[a]	Candidate gene functional category[b]	Details[b]
SNP0375	AUDPC, DI30, DIr	*Psat1g212920* chr1LG6: 362713996..362719342 (bp)	transport.unspecified cations	(at2g04305) Magnesium transporter CorA-like family protein
SNP2424	AUDPC, DI30, DIr	*Psat6g142560* chr6LG2: 282731302..282735281 (bp)	amino acid metabolism.degradation.se glycine-cysteine group.glycin	(at2g04305) Encodes a threonine aldolase, involved in threonine degradation to glycine
SNP2427				
SNP2428				
SNP2530	DI30	*Psat6g185920* chr6LG2:374643039..374646582 (bp)	RNA.regulation of transcription.C2H2 zinc finger family	(at1g34370) Encodes a putative nuclear Cys(2)His(2)-type zinc finger protein involved in H+ and Al3+ rhizotoxicity
SNP2540	AUDPC, DI30, DIr	*Psat6g189280* chr6LG2:381675809..381 (bp)	development.unspecified	(at1g34190) NAC domain containing protein 17
SNP2797	AUDPC, DI30, DIr	*Psat7g059960* chr7LG7: 99914774..99916577 (bp)	secondary metabolism.wax; misc.short chain dehydroge-nase/reductase (SDR)	(at1g24470) Encodes one of the two *Arabidopsis* homologues to YBR159w encoding a S. cerevisiae beta-ketoacyl reductase (KCR)

Note. AUDPC, area under disease progress curve; DI30, disease intensity at 30 d after infection; DIr, disease progress rate; SNP, single nucleotide polymorphism.
[a]From pea reference genome v1a (https://urgi.versailles.inra.fr/Species/Pisum).
[b]From Mercator4 v2.0 (https://www.plabipd.de/portal/mercator4).

lation between markers. Considering a LD decay threshold of $r^2 > 0.2$ as the boundaries of a genomic window adjacent to each significantly associated SNP location, none of the associated markers was in LD with any of the neighboring markers.

3.6 | Candidate genes relative expression analysis

The relative expression of the five candidate genes proposed directly through GWAS as genes involved in response to *Fop* infection (*Psat1g212920*, *Psa6g142560*, *Psa6g185920*, *Psat6g189280*, and *Psat7g059960*) was analyzed by RT-qPCR in disease response contrasting accessions. The relative expression was analyzed per accession by comparison with the respective noninoculated treatment along time (24 and 48 hai, 4 and 7 dai). The expression patterns per candidate gene and accession along time, as well as the significant differences detected, are shown in Supplemental Figure S7. In the resistant accession BGE19777, a downregulation at 48 hai was observed in the *Fop* inoculated plants in comparison with the noninoculated plants for *Psat1g212920* (0.78-fold change) and *Psat6g189280* (1.13-fold change). In the other resistant accession, PI 283593, *Psat7g059960* expression was upregulated at 48 hai in the *Fop* inoculated plants in comparison with the noninoculated plants (2.63-fold change). Also, in this resistant accession, *Psat6g142560* was constitutively highly expressed upon inoculation (1.68-fold change), with a downregulation just after 48 hai. For the other candidate genes, no differences were identified among the analyzed resistant and susceptible accessions at the studied time points after inoculation (Supplemental Figure S7).

4 | DISCUSSION

Fusarium wilt is a constraint to grass pea production and there is no current information on the genetic basis of resistance. A recent study showed that *Fop*, the causal agent of the phylogenetic close species pea, is also virulent on grass pea

(Sampaio et al., 2021a). By screening a grass pea worldwide collection of accessions against this pathogen infection, different sources of resistance were identified (Sampaio et al., 2021b). To unravel the genetic basis of the identified grass pea resistances, we conducted a GWAS, adding high-throughput genotypic information to this previously collected disease response data. Seventeen SNPs were identified as significantly associated with grass pea response to *Fop* race 2 ($-\log_{10}[p] \geq 4$). From these, 13 SNPs were commonly associated with the three disease response traits under analysis (AUDPC, DI30, and DIr), as expected for highly correlated traits (Sampaio et al., 2021b).

Each of the 17 significant SNP–trait associations explained merely a portion of the total genotypic variance, ranging from 7 to 29%, with an average of 13%. These results disclosed a grass pea oligogenic resistance to *Fop* race 2, corroborating the evidence of a quantitative nature of resistance revealed by the previous phenotypic characterization of this collection of accessions (Sampaio et al., 2021b). As revised by Sampaio et al. (2020) and Jha et al. (2020), an oligogenic resistance to fusarium wilt has been described in several other legumes. Examples are chickpea, for some races of *Fo* f. sp. *ciceris* (races 0, 1A, and 4) (Upadhyaya, Haware, et al., 1983; Upadhyaya, Smithson, et al., 1983; Singh et al., 1987; Tullu et al., 1999; Halila et al., 2009); lentil (*Lens culinaris* Medik.), for *Fo* f. sp. *lentis* (Kamboj et al., 1990); common bean, for *Fo* f. sp. *phaseoli* races 4 and 6 (Fall et al., 2001; Leitão et al., 2020); cowpea, for *Fo* f. sp. *tracheiphilum* (Wu et al., 2015); and pea, for this same *Fop* race 2 (McPhee et al., 2012). However, from the three known pea *Fop* race 2 resistance genes, *Fnw4.1* (located on pea chromosome 4) and *Fnw3.1* and *Fnw3.2* (on pea chromosome 5) (McPhee et al., 2012), only one marker–trait associations detected (SNP5205) might share the chromosomal location with a pea resistance gene. SNP5205 was located on grass pea LG IV through comparative mapping with the *L. culinaris* and *M. truncatula* genome, based on the existing macrosynteny among these related legume species (Santos et al., 2021). Grass pea LG IV is considered highly syntenic with pea chromosome 4 (Santos et al., 2021). The other associated SNPs detected in the present study, were located in pea chromosomes 1, 6 and 7 and grass pea LG I (or pea chromosome 1). Still, almost 50% of the grass pea markers significantly associated with resistance to *Fop*, including some of the most significantly associated markers, were considered "unmapped". This was due to lack of alignment with the pea genome or to the presence of more than a single hit with the same E-value, but they might also represent candidate genes with different chromosomic locations. All these unmapped markers were DArT-Seq markers, markers with a nucleotide sequence length of about 65 bp, hampering its accurate and specific location on the pea genome. Nevertheless, following the results of Santos et al. (2021) that observed higher linkage mapping synteny between grass pea and pea, than between grass pea and lentil or barrel medic (*Medicago truncatula* Gaertn.), pea seems to be the best phylogenetically related species to assembly grass pea markers along chromosomes.

The use of the pea reference genome to map the SNP markers and the assumption of high synteny between grass pea and pea genomes allowed the proposal of five candidate genes, directly obtained through GWAS, implicated in transport, amino acid metabolism, RNA (regulation of transcription), development, and secondary metabolism. To a successful host–pathogen colonization, several plant defense barriers must be transposed. One of those barriers is the plant cell wall. Cell wall integrity maintenance is important in preventing pathogen invasion, acting as a physical barrier but also as an antimicrobial compound reservoir (Miedes et al., 2014). Indeed, cell wall reinforcements were previously detected by histological analysis of selected *Fop* inoculated grass pea resistant accessions, from the same grass pea collection here used for GWAS, blocking the pathogen at the outer-root area (Sampaio et al., 2021b). Both magnesium transportation and biosynthesis of secondary metabolites as waxes are somehow related to the cell wall integrity maintenance but at different layers. At the middle lamella, the outermost layer of the cell wall, magnesium together with calcium makes pectin cell wall substances resistant to degradation. When adequate magnesium is available, fusarium wilt symptoms tend to be less severe due to the increase of resistance to extracellular pectin degrading enzymes of soil-borne fungi, as *Fo* (Huber & Jones, 2013). Although magnesium is in part responsible for cell wall integrity during *Fo* attacks, only through efficient transport it can reach the cell wall and contribute to the integrity. This may be one of the mechanisms present in the resistant accession BGE19777, where the candidate gene *Psat1g212920*, a magnesium transporter Cor-A-like family protein located in the membrane, was found downregulated 48 hai. At the secondary cell wall, waxes, together with cutin and lignins, are the major components allowing cell wall integrity maintenance (Houston et al., 2016). In a tolerant banana cultivar to *Fo* f. sp. *cubense*, genes related to wax biosynthesis were detected as upregulated in early stages of infection (Li et al., 2019). In this study, the proposed candidate gene involved in wax biosynthesis, *Psat7g059960*, was also found upregulated upon infection (48 hai) in the resistant accession PI283593, anticipating that wax biosynthesis may be important for this accession resistance.

Although the resistance mechanisms involved in fungus penetration inhibition are considered the main mechanisms for a successful resistance (Jiménez-Fernández et al., 2013), other important resistance mechanisms could act not only during penetration but also during pathogen progression. This seems to be the case of the mechanisms associated with *Psat6g142560*, constitutively highly expressed on the inoculated resistant accession PI283593 and downregulated just

after 48 hai. This gene is involved in amino acid biosynthesis and encodes for a threonine aldolase. During plant–pathogen interaction, both as a first response to pathogen attack and also during disease infection progression, amino acid metabolism alterations occur (Fagard et al., 2014). Alterations in amino acid metabolism were detected in chickpea upon infection with *Fo* f. sp. *ciceris*, namely in the susceptible cultivar (Rathod & Vakharia, 2011; Kumar et al., 2016). The decrease in the levels of several amino acids in the susceptible cultivars, suggests that *Fo* utilizes them for its establishment, development, and proliferation (Kumar et al., 2016). However, the exact role of such amino acid modifications in pathogen defense remains unknown. Threonine aldolase has been reported to play a role in seed nutritional quality (Jander et al., 2004), but its involvement in plant-pathogen defense was never reported so far. Nevertheless, its enzymatic function is inducible by methyl jasmonate (Broeckling et al., 2005), which plays an important regulatory role in plant defense (Wasternack, 2007).

When *Fop* is already growing in the upper part of the vascular vessels, resistance mechanisms to avoid dramatic wilting can still operate. One possible candidate gene involved in the later stages of *Fop* resistance is the *Psat6g189280* gene, downregulated at 48 hai on the resistant accession BGE19777 in the present study and encoding a NAC domain-containing protein 17 (NAC017) known to be involved in development. In *Arabidopsis*, a single mutant of *nac017* accelerates cell death and leaf senescence, indicating that NAC017 (together with NAC082 and NAC090) governs positive-to-negative regulatory shift in leaf senescence (Kim et al., 2018). Although NAC017 is not involved directly in *Fo* resistance, leaf senesce is one of the symptoms caused by *Fo* infection. This suggests that although never reported in fusarium wilt resistance, NAC017 can be somehow related to it by maintaining plant integrity.

Psat6g185920, another candidate gene, encodes a Cys(2)His(2)-type zinc finger protein. Although in *Arabidopsis thaliana* this protein is involved in proton and aluminum rhizotoxicity response (Sawaki et al., 2009), in *Nicotiana benthamiana* it has been related with plant defense, acting as a regulator of SSCut, a plant elicitor that induces plant immunity through cell death during infection of the soil-borne oomycete *Phytophthora nicotianae* (Zhang et al., 2016). Nevertheless, no significant differences were detected on this candidate gene expression. Different resistant accessions may have different genetic basis of resistance, resulting into different candidate gene expression patterns across time, and so we do not exclude that differences in the candidate genes expression could be higher by analyzing different time points after infection or different resistant accessions. Indeed, the phenotypic characterization was performed until 30 dai, offering a wide range of time points where these genes can be differentially expressed. Furthermore, the resistant accessions used for RT-qPCR analysis were characterized by low AUDPC and DIr (Sampaio et al., 2021b) suggesting that in these accessions the infection started early but have progressed very slow. If accessions with different resistance mechanisms, where disease symptoms start late although progressing fast (low AUDPC and high DIr) have been included, perhaps different gene expression patterns could have been observed.

The assembly and annotation of the grass pea full reference genome are still ongoing (Emmrich et al., 2020), imposing extra challenges on the interpretation of grass pea GWAS results. If a complete assembled genome would be available, a higher percentage of the screened SNP markers would have been allocated to a grass pea chromosome physical position, allowing higher identification of candidate genes. In this study, nucleotide-binding, leucine-rich-repeat genes, one of the major disease resistance gene types, were not detected. Nevertheless, this result is in line with the ones obtained in other legume species, where no nucleotide-binding, leucine-rich-repeat genes have been reported as involved in fusarium wilt resistance (Gupta et al., 2017; Chang et al., 2019; Leitão et al., 2020).

In all associated SNPs, the favorable allele (conferring increased resistance) was the most frequent in both seed color (light and dark) and seed size (large and small) groups of accessions, suggesting that most accessions have many favorable alleles, although lighter and larger accessions presented higher frequency levels of favorable alleles. These results concur with what was observed during the grass pea accessions phenotypic characterization, with lighter and larger accessions looking consistently more resistant to *Fop* (Sampaio et al., 2021b). Asian and Ethiopian accessions, characterized by darker and smaller seeds (Przybylska et al., 2000), presented in general the lower SNP beneficial alleles frequency. Ethiopian grass pea accessions were also considered the most susceptible in the previous phenotypic characterization to *Fop* infection, whereas Asian accessions revealed a considerable range of responses, including resistance and susceptibility (Sampaio et al., 2021b). Our results suggest that it might be possible to increase the resistance levels of Ethiopian and Asian accessions by introgression of the present identified resistant alleles in these grass pea populations. European and especially Northern African accessions revealed to be promising sources of resistance alleles, with high frequencies of the favorable allele in almost all the associated SNPs. However, to improve Asian and Ethiopian grass pea populations, frequently characterized by dark and smaller seeds, accessions with the same morphological seed types would be optimal to integrate into cross-breeding programs. Interestingly, by principal component analysis and STRUCTURE analyses, smaller and darker seeded grass pea accessions were characterized by higher diversity and higher genetic admixtures, besides a wider range of disease responses (Sampaio et al.,

2021b), when compared with the lighter and larger seeded accessions. This higher diversity makes this particular ecotype of smaller and darker seeds a very promising source of interesting trait combinations for future breeding. Indeed, several dark and small seed accessions have been identified as resistant to fusarium wilt and good candidates for resistance breeding within this ecotype. Examples may be the Asian accession PI426897 (belonging to genetic cluster B) and the Ethiopian accession PI226948 (belonging to genetic cluster A with admixture). These are both characterized by darker and smaller seeds, lower disease symptoms (Sampaio et al., 2021b), and the presence of the favorable allele for almost all the associated SNPs, being considered valuable sources of resistance to be included in the dark, smaller seed breeding programs. Only by considering the associated SNP with the highest effect (SNP3907) on *Fop* resistance, selection in favor of the beneficial allelic variant "G", will substantially reduce susceptibility for all the traits measured (24.5% in AUDPC, 26.9% in DI30, and 29.1% in DIr).

Phenotypic field evaluation of the grass pea worldwide collection to fusarium wilt is also needed in the future to confirm the maintenance at field level of the marker–trait associations here revealed under controlled environmental conditions using a trimmed-root inoculation approach.

With the present study, by exploring the natural variation present in a worldwide grass pea collection, several genomic regions controlling resistance to *Fop* race 2 were unraveled reinforcing the usefulness of this association panel and the comparative mapping approach here applied. The favorable SNP alleles and putative candidate genes here identified constitute important tools to assist precision breeding for fusarium wilt resistance in grass pea. These might be potentially also valuable for pea breeding if crossability barriers are overcome. Special efforts should focus on SNPs with the strongest associations and/or SNPs, which alleles confer higher resistance levels.

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Ana Margarida Sampaio: Data curation; Formal analysis; Investigation; Methodology; Software; Validation; Visualization; Writing-original draft; Writing-review & editing. Mara Lisa Alves: Formal analysis; Methodology; Software; Validation; Writing-review & editing. Priscila Pereira: Data curation; Investigation. Ehsan Valiollahi: Formal analysis; Software; Writing-review & editing. Carmen Santos: Formal analysis; Methodology; Software; Writing-review & editing. Zlatko Šatović: Formal analysis; Software; Writing-review & editing. Diego Rubiales: Supervision; Validation; Writing-review & editing. Susana de Sousa Araújo: Methodology; Supervision; Validation; Writing-review & editing. Fred van Eeuwijk: Methodology; Software; Validation; Writing-review & editing. Maria Carlota Vaz Patto: Conceptualization; Funding acquisition; Project administration; Resources; Validation; Writing-original draft; Writing-review & editing.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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